INFLUENCE OF DIETARY STARCH INCLUSION ON CECAL ENVIRONMENT AND MICROBIAL POPULATIONS IN HORSES

A Thesis

by

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Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

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August 2013

Major Subject: Animal Science

ABSTRACT

Previous research has documented shifts in microbial hindgut populations resulting from dietary starch inclusion, and recent evidence indicates only 30% of equine cecal contents has been cultured successfully. Next generation sequencing (NGS) techniques allows detection of new species previously undetected. Therefore, the objective of this study was to determine community profiles equine cecal microbiota in response to abrupt dietary starch inclusion. Seven cecally cannulated Quarter horse geldings (497 to 580 kg) were utilized in a crossover design with two 28 d periods and a 28 d washout between each. Horses were randomly assigned to dietary treatments consisting of commercial concentrate offered individually at either 0.6% (LS) or 1.2% BW (HS**;** as fed) daily divided into 2 meals at 12 h intervals. Prior to start of each period horses were allowed ad libitum access to coastal bermudagrass (*Cynodon dactylon*) hay and concentrate was fed on d 1 with no adaptation. Samples of cecal fluid were collected on d 1 prior to 0 h and 3, 6, 9, and 12 h post morning meal and on d 1, 2, 3, and 7 at 6 h post morning meal. Cecal pH was determined immediately and a samples of cecal fluid were stored. Genomic DNA was extracted and the V4-V6 segment of 16s rRNA gene was PCR amplified using universal Eubacterial primers 530Fand 1100R and sequenced on the Roche 454 FLX platform. The reads were denoised, chimera checked, and Operational Taxonomic Units (OTUs) were identified using the reference Ribosomal Database Project 16S rRNA dataset. Data were analyzed using PROC MIXED procedure of SAS. Bacterial phyla were largely unaffected by dietary treatment for the first 12 h after the initial concentrate meal except for *Verrucomicrobia* which was

greater in LS horses ($P \leq 0.04$). Regardless of treatment, *Bacteriodetes* increased ($P \leq 0.02$) over the first 12 h following initial addition of dietary starch. Adaptation to dietary treatments over 7 d resulted in decreased numbers of *Tenericutes* ($P \leq 0.07$) in HS horses compared to LS. Cecal environment and microbial populations were altered after abrupt and long term exposure to dietary starch.

DEDICATION

This thesis is dedicated to my grandmother, Bebe, and my grandfather, Lad, for their love, support, and encouragement throughout my academic career.

ACKNOWLEDGEMENTS

I would like to thank my chair, Dr. Josie Coverdale, as well as my committee members, Dr. Tryon Wickersham, Dr. William Pinchak, and Dr. Jan Janecka, for their contributions to this study and for their guidance and support during my time at Texas A&M University.

TABLE OF CONTENTS

Page

LIST OF FIGURES

Page

LIST OF TABLES

Page

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Introduction

Abrupt influx of dietary starch affects fermentation dynamics in the equine cecum, resulting in alterations in pH, microbial ecosystems and their metabolites. Changing community structure of the equine microbiome with addition of dietary starch found in concentrate rations has been linked to increased incidence of gastrointestinal upset and one of the predisposing factors for laminitis (Milinovich et al., 2005; Werners et al., 2005; Costa and Weese, 2012). Performance horses are often subjected to abrupt changes in diet, specifically inclusion of high concentrate rations with little adaptation. Typical diets fed to horses in light and moderate work can range from 0.5% to 1.5% BW in the concentrate portion of the ration. Increased concentrate in the digestive system can overwhelm the starch digestion capacity of the small intestine, allowing starch to escape enzymatic digestion and absorption. Undigested and unabsorbed starch in the small intestine enters the hindgut and leads to rapid cecal fermentation.

Starch fermentation, by microorganisms in the cecum, alters volatile fatty acid (VFA) concentrations, which are important sources of energy for the horse. However, it also causes a decrease in cecal pH, negatively affecting gastrointestinal health (Argenzio et al., 1974b; Willard et al., 1977; de Fombelle et al., 2001). Abrupt changes in diet composition, specifically with inclusion of dietary starch, increase total viable bacteria in the cecum of horses (de Fombelle et al., 2001). Similar changes in bacterial counts have

been observed in other species. Ruminants have served as a model for microbial research in relation to meal feeding due to the similarities in fermentation patterns in the rumen and monogastric cecum (Kern et al., 1974). Data collected from microbial research in ruminant studies have served as a useful tool for determining taxa present in the similar environment of the equine cecum (Kern et al., 1974; Allison et al., 1974; Fernando et al., 2010; Petri et al., 2012). However, a more thorough investigation of microbial community structure and detection of unclassified novel species thriving in the equine cecum require more detailed evaluation.

Functional Anatomy of the Equine Large Intestine

Horses possess a relatively large hindgut, which comprises 60% of the total tract, and rely heavily on fermentation for the release of dietary energy via VFA (Argenzio et al., 1974b). Before ingested material is fermented in the hindgut, the stomach aids in mechanical and enzymatic breakdown of the feedstuff. Following digestion in the stomach, the duodenum of the small intestine serves as a secondary site of enzymatic digestion of the feedstuff. After the digesta enters the small intestine, it is stabilized to a pH of 7. As digesta moves into the cecum from the ileum, the final segment of the small intestine, it is stabilized at a pH of 6.5 to 7. Transit time for ingested material to reach the cecum has been shown to be as fast as 90 min (Howell and Cupps, 1949). The ileocecal junction acts as a barrier to prevent material from entering back into the ileum (Cottrell et al., 1998).

The cecum is a blind-ended vat ranging in length from 1.5 to 2 m and has a capacity of approximately 30 l (Argenizio, 1974a). Both a fluid and particulate phase

are present here, but are not separated into distinct layers as in the rumen. Non-rythmic haustal movements and contractions force material to move into the apex and exit the cecum cranially (Sellers and Lowe, 1986). The cecum, the main site of anaerobic fermentation in the equine, maintains an internal temperature between 37 and 40˚C. Bacteria, responsible for the production of short chain VFAs such as acetic, propionic, and butyric acid from dietary carbohydrate substrates, convert dietary and endogenous nitrogenous compounds into ammonia and microbial crude protein, and synthesize B vitamins (Stevens and Hume, 1998). Peristaltic movement forces digesta into the right ventral colon from the cecum within 3 h. A continuous flux and reflux between cecum and proximal colon exists, leading to cecal retention of fluid and the rejection of particles (Argenzio et al., 1974a). An estimated 60 to 70% of horses' available energy is derived from the absorption of VFAs in the large intestine (Argenzio et al., 1974b; 1975). The colon serves as the last segment to absorb water and products of fermentation before excretion in feces.

Physiological and Microbial Changes Dependent on Substrate

Incorporating concentrate meals addresses increased energy requirements of work and other physiological functions and improves athletic performance of the horse. Current management strategies involve meal feedings and contradict continuous grazing nutritional ecology of the native horse, which relied solely on a forage based diet. Modern diets are composed primarily of structural and non-structural carbohydrates (NSC), with a substantial portion of the later in the form of starch. Increased ingestion of concentrate can overwhelm the capacity of small intestinal enzymatic starch digestion,

allowing it to enter the cecum (Potter et al., 1992). Starch overload occurs when concentration of starch in the diet exceeds 4.0 g/kg BW per meal and significantly exceeds the capacity of small intestine enzymes to digest starch preventing its absorption (Potter et al., 1992). This inefficiency can be blamed partially on the relatively short length of the equine small intestine. Therefore, the type of diet chosen and volume of meal offered dictate substrates that reach the cecum and are available for fermentation. In particular, the addition of dietary starch to the diet is associated with decrease of cecal pH and increase of lactate (de Fombelle et al., 2001).

Cecal pH. Changes in cecal pH are dependent on the substrate fed. Inhibition of cellulose digestion by fibrolytic species occurs when the pH falls below 6.0 (Hoover, 2010). Willard et al. (1977) reported cecal pH was lowest between 4, 5, and 6 h post concentrate meal feeding when compared to a hay diet. Decreases in pH have also been observed in ruminants administered feedlot rations high in rapidly fermentable carbohydrates. Lactic acid production from starch fermentation results in lower rumen pH and inhibits digestion of cellulose in ruminants (Allen, 1997). A decrease in cecal pH effects production of acetate, propionate, and butyrate (Allison et al., 1974).

Volatile Fatty Acids. End products of fermentation include VFAs, specifically acetic acid, propionic acid, and butyric acid. Julliand et al. (2001) noted a relationship between VFA and pH in horses fed a diet supplemented with barley. Horses fed barley had higher total VFAs, propionate, and lactate concentrations accompanied by a decrease in cecal and right ventral colonic pH. Similarly, Willard, (1997) fed horses concentrate diets and observed lower percentages of cecal acetate and higher propionate

compared to horses fed hay diets . Feeding a diet containing more than 50% concentrate can lead to a rise of molar proportions of propionate, butyrate, valerate, isovalerate, and a decrease in acetate in the cecum (Hintz et al., 1971). Hungate et al. (1996) concluded that differences in molar percentages of VFAs resulted directly from alterations in microbial populations. Julliand et al. (2001) observed a decrease in concentrations of cellulolytic bacteria with addition of dietary barley. This resulted in decreased molar percentage of acetate, and a reduction of the [(acetate + butyrate)/propionate] ratio. Lactate can also be produced from fermentation of glucose and converted to propionate via the acrylate pathway by *Lactobacillus* spp, which increase in the cecum after an abrupt change to a diet high in starch (de Fombelle et al., 2001). Decreased cecal pH results from an increase in lactate and propionate levels in response to rapid fermentation in the cecum, and can lead to irritation of the gut wall (Prins and Van Der Meer, 1976).

Microbial Populations. The equine cecum and colon harbor diverse microflora estimated at 0.5×10^9 to 5×10^9/g (Frape, 2010). The cecum is densely populated with bacteria, and 40 to 45% of cecal microbes are strict anaerobes (McCreery et al., 1971; Kern et al., 1973). Previous research has shown that diet alters cecal microbial numbers (Grubb and Dehority, 1975; Kern et al., 1974). After a dietary change, microbes adapt to the substrate offered and 28 d has been reported in sheep for complete adaptation (Lloyd et al., 1956). However, factors including abruptness of diet change play a role in determining adaptation length (Lloyd et al, 1956; Hall and Woolfolk, 1952).

The ruminant animal has served as a model for determining taxa present in the cecum of horses because many bacteria found in the respective host systems are similar

and thrive under relatively comparable environments. Cannulation procedures in horses are time consuming, labor intensive, and costly, which has led researchers to investigate the rumen as a model. The hindgut and rumen environment are often compared to a rainforest, where many species thrive and engage in symbiotic relationships. They digest primarily carbohydrates, composed of cellulose, hemicellulose, pectin, starch, and sugars and metabolites are excreted for metabolic use by other bacteria (Van Soest, 1982). Dietary substrates directly affect bacteria in each system. Fructans exist in many ruminant and equine diets due to their role as storage carbohydrates in grass. Fructans have been reported to escape digestion in the small intestine leading to fermentation in the hindgut (Crawford et al., 2007).

Cellulose, the most abundant structural polysaccharide constituent of the plant cell wall, escapes enzyme digestion in the small intestine due to the enzymatic inability to cleave β 1, 4 bonds. Because of this enzymatic deficiency in the small intestine, dietary cellulose utilization is dependent on specific cellulolytic microbes in the cecum. Cellulolytic bacteria secrete cellulase to break β 1, 4 glycosidic bonds in cellulose by directly attaching to the fibrous portion of the plant material. To date, major cultured species include *Fibrobacter succinogenes, Ruminococcus albus, Ruminococcus flavefaciens,* and *Butyrivibrio fibrisolvens*. *Ruminococcus* spp digest cellulose and hemicellulose although glucose and cellobiose are favored substrates for growth (Yokoyama and Johnson, 1988).

Changes in bacterial fermentation result in a decrease in cecal pH and an altered VFA ratio when cereal concentrates are added to the diet. Major cultured amylolytic

species include *Bacteriodes amylophilus, Streptococcus bovis, Succinimonas amylolytica,* and *Bacteriodes ruminocola* (Yokoyama and Johnson, 1988). Proteolytic bacteria synthesize endopeptidades and exopeptidases and comprise 19.7% of bacteria in the cecum. Major cultured species include *Bacteriodes amylophilus, Bacteriodes ruminocola, Butyrivibrio fibrisolens,* and *Streptococcus bovis* (Yokoyama and Johnson, 1988). Depending on metabolic pathway and whether the bacteria engages in cellulolytic, amylolytic, or proteolytic tendencies, microbes have the capability to use nutrients to produce metabolites that can benefit the microbe itself and/or other microbes in the system as well as the host.

The microbial system is dynamic, indicating microorganisms adapt with time to their environment (Sonnleitner et al., 1998). Microbes within an environment differ in intracellular composition to survive in the given environmental condition. Specifically, microbes can differ in enzyme expression levels or catabolism pathways (Sonnleitner et al., 1998). Microbes not possessing essential enzymes for survival in a particular environment will die.

The length of diet adaptation of the host can promote or impair health status of the animal depending on length of time and amount/type of substrate offered (Brown et al., 2006). Studies in which feedlot cattle were transitioned from 55 to 90% in dietary concentrate in 14 d or less observed reduced performance during adaptation periods. Similarly, Burrin et al. (1988) observed a 60% reduction in dry matter intake (DMI) on d 4 after animals were switched from a 75% concentrate diet to a 95% offered a libitum. Studies in ruminants have also noted increases and decreases in bacterial taxa during the

adaptation period. Klieve et al. (2003) observed an increase in *S. bovis* and *M. elsdenii* and a decrease in *B. fibrosolvens* in one animal transitioned from forage to a 45% barley diet. Adapting the the rumen microbiome towards a greater number of starch fermenting bacteria has been accomplished by utilizing step up diets over a 3 to 4 week period (Goad et al., 1998; Bevans et al., 2005). Specifically, adequate time is needed to adapt lactate metabolizing species, so the conversion of lactate to propionate can be performed. Microbial changes occur during the adaptation period to a diet; however, little work has been done determining the amount of time for microbes to adapt to substrate offered and even fewer studies have investigated adaptation in the horse.

Determining Microbial Populations

The majority of equine gastrointestinal microbial research to date has relied heavily on *in vitro* culture based methods for quantifying viable counts of bacteria. Traditional culture based techniques have capability to isolate anaerobic bacteria directly from environmental samples using a selective or differential agar media. Agar mediums are infused with specific nutrients required for the growth of organisms. Mangels et al. (1989) determined Anaerobe Systems prereduced and sterilized Brucella PRAS agar was superior to competitors Brucella agars for enumerating anaerobes. More colonies appeared earlier and were larger and easier to detect due to the packaging procedure that eliminates deterioration and contamination with O_2 until the time of use (Mangels et al., 1989). Other studies use agar infused with varying levels of glucose, starch, lactate, and trypticase for enumeration of total culturable bacteria (Mackie and Wilkins, 1988).

When targeting cellulolytic bacteria specifically, media inoculated with cellulose and cellobiose are used (Julliand et al., 1999).

After the appropriate agar is determined for use, the sample is serial diluted, plated on the chosen agar, and incubated. Colonies are selected and subjected to gram staining for determination of species. Most culture based studies performed in horses give a representation of the normal flora in the gastrointestinal tract, but low animal numbers and ability to obtain only 30% of culturable bacteria from the cecum in past studies must be taken into consideration when reviewing previous literature (Mackie and Wilkins, 1988).

Early studies by Kern et al. (1973) compared microbial populations in the cecum of ponies and rumen of steers in response to diet. Four cecally cannulated Shetland ponies and 4 steers with rumen cannulas were used in a simultaneous 4 × 4 Latin square experiment. Bacteria counts were determined using a non-selective agar medium and anaerobic roll tubes (Hungate, 1950). Cellulolytic bacteria per g ingesta were similar in the cecum and rumen regardless of diet. Major known species identified were *Steptococcus bovis, Streptococcus equinus,* and known genera were *Lactobacillus, Bacteriodes, Propionibacterium,* and *Borrelia*.

A later study by Kern et al. (1974) evaluated bacterial counts in the cecum of geldings and the rumen of steers in response to 7% CP diet of long timothy hay. Diets were fed 30 d prior to euthanasia to ensure microbial adaptation. Contents from the ileum, cecum, and colon were collected from the horses; whereas, contents from the rumen and abomasum, pyloric and fundic regions, were collected from steers. The

greatest numbers of cellulolytic bacteria were found in the pony cecum and the steer rumen. Overall, the rumen contained greater total viable bacteria counts compared to the pony cecum. Cellulolytic bacteria counts were greater in the rumen compared to the cecum (70.3×10^6 g/content and 43.0×10^6 g/content, respectively). When comparing anatomical segments of the equine gastrointestinal tract, cellulolytic bacteria were found in the highest concentrations in the cecum (43.0×10^6 g/content, followed by the colon (25.6×10^6 g/content), and lastly stomach (0.0003×10^6 g/content. Due to large numbers of unculturable cecal organisms, the diversity of cellulolytic bacteria and their role in fermentation must be further researched.

Goodson et al. (1988) observed greater total anaerobic bacterial counts in the cecum of one pony when diets were switched from alfalfa hay (13.5% CP) to a diet composed of 86.7% ground corn and 13.3% soybean meal. The mean viable count when concentrate was included in the diet was 30.07×10^9/g, which was greater than 5.28×10^9/g detected from the hay diet. This change in population occurred 48 h after the diet was switched from forage to concentrate. During the period when hay was fed, starch utilizing bacteria averaged 73.1% of the total anaerobes compared to 85.2% when concentrate was fed. Starch utilizing bacteria increased to 92.2% of the total bacterial numbers 24 h after the initial concentrate meal and eventually decreased to 87.5% by 48 hr. Lactate-utilizing bacteria did not change until d 3 to 7, when they rapidly increased to 69.2% of the total bacterial numbers (Goodson et al., 1988). Techniques for enumeration used by Goodson et al. (1988) included isolating bacteria on a xylose medium. Growth of strains appeared slow and overestimated population growth by 6 to 7% of the total

culturable count according to techniques used to Dehority and Grubb (1976), which were compared by Goodson et al., (1988). Therefore, these results indicate that selecting appropriate culture media for the vast number of microorganisms can prove to be challenging as well as laborious and costly.

Pioneering Application of 16S rRNA Gene to Microbial Studies

The emerging field of metagenomics has led to the ability to analyze genomes of microorganisms in different environments using culture independent methods (Handelsman, 2004). Over the past century, metagenomic research has yielded information pertaining to measurement of structure and dynamics of microbial communities and interactions between microbes as well as the host (Weinstock, 2012). Results from The Human Microbiome Project and The Human Genome Project concluded that more than 3 million microbial genes reside in the human gut. This number is 100 fold higher than genes found in the human genome alone (Qin et al., 2010). The complexity and abundance of microorganisms has posed a challenge to researchers due to inability of certain species to grow in pure culture. Many novel species require specific growth conditions that can only be achieved in vivo. New insights into culture-independent methods have revealed a more efficient and thorough way to identify microorganisms by investigating their genetic makeup.

The 16S small ribosomal subunit gene has been an important marker gene over the past quarter century and has contributed to findings in microbial ecology and metagenomics. In study of microbial evolution, the 16s gene is notably known for its sequence conservation and a domain structure with variable evolutionary rates (Tringe

and Hugenholtz, 2008). The gene is commonly used to identify constituents of microbial communities and to design analysis methods for classifying bacteria and archae (DeSantis et al., 2006). Many studies have targeted 16S for community composition profiling before complete metagenomic analysis (Tringe and Hugenholtz, 2008).

Previous studies using culture independent methods of classifying bacteria primarily targeted microorganisms in equine feces (Morotomi et al., 2002; Endo et al., 2008; Steelman et al., 2012), while other studies have harvested gastrointestinal contents from horses following euthanasia (Lin and Stahl, 1995; Daly et al., 2006) or by gastric biopsy (Perkins et al., 2012). These studies serve as a model for how the 16s gene is used to target organisms in different anatomical regions of the equine gastrointestinal tract using cloning, polymerase chain reaction (PCR), and fluorescent in situ hybridization (FISH).

Lin and Stahl, (1995) examined gastrointestinal contents of one pony and quantified *Fibrobacter* diversity using 16S rRNA targeted oligonucleotide probes. Probes were designed to complement the targeted 16S rRNA region that codes for genus, species, and subspecies level. Samples from the ileum, cecum, and colon were harvested following euthanasia and stored at -85˚C until nucleic acid extraction was performed. For quantification purposes, total rRNA abundance was determined using a Universal probe complimentary to all characterized 16S rRNAs (Stahl et al., 1988). To determine diversity between genus, species, and subspecies, DNA was extracted and PCR amplified using Universal primers as described by Lane et al. (1991). Phylogenetic trees were constructed to show diversity and similarity between *Fibrobacter* species.

After hybridization, 12% of total rRNA isolated from the cecal sample accounted for *Fibrobacter* rRNA. The colon contained 4% of total *Fibrobacter* rRNA, which was undetectable in the ileum. Prior to PCR amplification of targeted sequences, products were cloned and sequences were determined. Sequence identification revealed that all clones were related to the *Fibrobacter* genus. Two species, *F. succinogenes* and *F. intestinalis* were identified.

A similar study performed by Daly et al. (2000) examined molecular diversity in different segments of the large intestine of five grass fed horses following euthanasia. Total genomic DNA was extracted and 720 bp of the 16S rRNA gene were amplified using universal eubacterial primers P3-Mod and PC5. Amplified products were cloned into vector plasmids and partial DNA sequences were compared to the EMBL and GenBank databases using BLAST procedures. Approximately 272 clone sequences were classified into 168 operational taxonomic units (OTUs) on > 97% similarity. Approximately 77% of the total number of sequences showed similarity values in the range of 90-97%, and12% of sequences showed less than 90% homology, while only 11% corresponded to previously recorded sequences in the database at a level of 97% similarity. Based on phylogenetic analysis, low G+C Gram-positive bacteria comprised 72% of the total number of sequences analyzed. Clostridium-Firmicutes-Bacteriodes phylum category accounted for 20% of the sequences, while only 8% belonged to the remaining phyla *Spirochaetes.* High G+C Gram-positive bacteria and *Proteobacteria* made up < 1% of the total population. No sequences relating to *Fibrobacter succinogenes* or *Ruminococcus albus* were detected in this study, which conflicts with

results reported by Julliand et al., (1999) who determined that *Fibrobacter succinogenes* accounted for 12% of total rRNA extracted from the cecum. Similarly and Lin and Stahl, (1995) reported that *Ruminoccoccus flavefacienes* was the predominant cellulolytic species in the ceceum, compromising 9% of the total 16S rRNA. The amount of variation between studies could be attributed to PCR bias or variation in sampling procedures.

In addition to introduced bias within studies, few studies have identified novel sequences that do not correspond with those recorded in public databases. The lack of information of sequence identity in the databases requires more effort of researchers to accurately characterize what groups of bacteria are present in this dynamic system.

The 16S rRNA subunit has also been used for studies in which laminitis was induced by oligofructose administration. Fermentation of carbohydrates composed of starch, sugars, and fructan, results in the overgrowth of gram-positive bacteria and a decrease in gram-negative species, a relatively large component in the composition of hindgut microbiota (Garner et al., 1975; Kronfield and Harris, 2003; Bailey et al., 2003b). The rapid lysis of gram-negative species results in the release of endotoxins into the lumen of the hindgut. Lipopolysaccharide, an endotoxin constituent of the outer membrane of gram-negative bacteria, has been associated with the cascade leading to colic (Senior et al., 2011) and laminitis (Sprouse et al., 1987; Garner et al., 1978; Bailey et al., 2004; Onishi et al., 2012) particularly resulting in impaired circulation to the lower limb. The notable characteristic of lipopolysaccharide includes a hydrophobic lipid A moiety, which is specific to solely gram-negative bacteria (Moore and Morris 1992). The

lipid A moiety leads to endotoxic shock after endotoxins diffuse across the lumen of the cecum, which is compromised due to irritation by lactic acid produced by many of the gram-positive species. Symptoms of colic are likely apparent at this stage and result from irritation of gut mucosa by lactate. Endotoxins are released into the bloodstream after passing across the cecum wall, and trigger inflammation of the lamellar ischemia thought to lead to acute laminitis (Hood, 1993).

All bacterial derived bioactive metabolites, vasoactive amines, and matrix metalloproteinases (MMPs) have produced physiological signs of inflammation in the hoof (Bailey et al., 2001; Onishi et al., 2012). Vasoactive amines are produced from decarboxylation of amino acids by proteolytic bacteria (Rice et al., 1976). These amines are produced by gram-positive bacteria including *Streptococci* and *Lactobacilli* and have been reported in high concentrations in the cecum following addition of dietary starch (Bailey et al., 2002 and 2003a). Once released into circulation, amines affect digital vasoconstriction and interact with serotonin and adrenoreceptors (Bailey et al., 2002). This has been shown in both *in vitro* and *in vivo* studies (Bailey et al., 2000; Elliott et al., 2003). These factors can lead to the detachment of the distal phalanx from hoof wall, in severe cases, bone can displace, rotate, and penetrate the sole resulting in severe lameness (Milinovich et al., 2008). Laminitis is a multifactorial disease and determining exact trigger factors and their interactions proves to be a challenge.

Onishi et al. (2012) detected and enumerated gram-positive and gram-negative bacteria following administration of either corn starch or oligofructose to induce laminitis in 12 horses using PCR amplification of the V3 region of the 16S rRNA gene

and Denaturing Gradient Gel Electrophoresis (DGGE) band identification procedures. The DGGE assay uses gel electrophoresis of PCR amplified gene segments to examine microbial diversity (Deng et al., 2008). These results were compared to 8 control horses without induced laminitis. Samples of cecal digesta were obtained after horses were euthanized when an Obel grade lameness score of 2 was reached in treated horses. Genomic DNA was extracted, PCR amplified, subjected to DGGE analysis, and sequenced. Results indicated gram-positive bacteria increased following administration of cornstarch or oligofructose, oligofructose resulted in greater numbers. However, it is important to note that DGGE can only identify microbes represented at greater than 1% of the community, making it impossible to characterize taxa present in a lower abundance (Muyzer et al., 1993). Other studies have reported an increase in gram-positive species prior to the induction of laminitis. *S. lutetiensis,* a gram-positive bacterium, has been reported as the dominant species prior to the onset of laminitis (Milinovich et al., 2008)..

Laminitis accounts for 7.5 to 15.7% of all lameness issues in horses (USDA, 2000). It is a disease with little options after onset, and preventative strategies are desperately needed. The specific role of the hindgut and its microbial community in the pathology of laminitis is uncertain. Therefore, studies with a larger coverage of sequences in samples must be performed in order to determine the exact role of gram-positive bacteria, specifically *Streptococcus* spp., in starch fermentation in the hindgut. A more in depth analysis of bacteria present in the cecum can serve as a model for future studies investigating the trigger factors associated with the onset of laminitis.

Impact of 454 Sequencing

For more than 25 years, Sanger sequencing dominated the DNA sequencing field due to the ability to sequence whole or targeted regions of organism genomes. The downside of Sanger sequencing lies in isolation steps prior to sequencing that require library construction of bacterial clones, plating and growing bacteria, and cloning DNA fragments into vectors (Rogers and Venter, 2005). The latter steps require amplification and purification of template strands and electrophoresis of product to detect sequences. Overall, cloning is the most time consuming step. The reduction in cost and time of DNA sequencing in high throughput machines has made second generation sequencers, specifically the Roche (454) Genome Sequencer (GS) and Illumina, quite popular (Qin et al., 2010; Peterson et al., 2009).

Mechanism. 454 sequencing uses pyrophosphate base sequencing to sequence over one million DNA fragments simultaneously, a term more commonly referred to as "massively parallel sequencing." It also produces larger reads in excess of 400 bp (McPherson, 2009) in length with higher coverage than traditional Sanger methods. The 454 sequencing also eliminates bias associated with cloning DNA fragments. All second generation sequencers have novel characteristics of sequencing millions of bases in a shorter amount of time than Sanger methods and light optic detection of individual nucleotides. Sogin et al. (2006) targeted the V6 region of the 16s gene and amplified the region using universal bacterial primers to determine sequence tags in 8 environmental samples. One run in the 454 sequencer generated a total of 118,000 sequences, which is more output derived from any study using Sanger based methods. These enhancements

in efficiency and throughput will help facilitate taxonomic placement of sequences in gene records in public databases.

In order for sequencing reactions to take place, adapters ligate to both ends of fragmented genomic DNA. These adapters allow for conjoining of a bead to a DNA fragment, and are performed under conditions that specifically allow only one fragment of DNA to bind to one bead (Rogers and Venter, 2005). The DNA is amplified using the PCR procedure, which happens in an emulsion to keep amplification pure. The mechanism of PCR uses a reaction in oil emersion and to synthesize a specific DNA fragment from a single stranded template sequence (Arnheim and Erlich, 1992). Concluding the PCR reaction, there will be an estimated 10 million copies of the initial DNA fragment on each bead (Rogers and Venter, 2005). Prior to the sequencing reaction, these beads containing the amplified fragment are loaded onto a picolitre plate containing millions of wells, and one bead is distributed in each well. The wells are sized appropriately so only one bead is permitted. Each well serves as an individual reaction sites where enzymatic activity takes place. Nucleotides are washed over the plate and individual bases are assigned a color that is detected as each nucleotide on the complimentary chain finds its match on the template strand. As color assigned nucleotides are detected, wells emit light that is captured by a fiber optic bundle as the growing strand is sequenced. A wash containing apyrase is used to eliminate carryover effect from nucleotides left in the wells. Sequences are then generated with an accuracy rate of 99% or greater (Margulies et al., 2005).

Although second generation sequencers including Roche 454 have a higher coverage of reads at a lower cost, more error exists pertaining to the accuracy of each individual read length, particularly in genomic regions where there are repetitive sequences containing homopolymers. Insertions are the most common errors, followed by deletions, mismatches, and ambiguous base calls, respectively (Huse et al., 2007). The presence of homopolymers and their orientation in the sequence as well as sequence lengths themselves are other contributing factors leading to error rates (Gilles et al., 2011). Additionally, PCR substitutions and chimeras have been reported as a source of error leading to noise in the dataset (Quince et al., 2011). Overall read lengths are also shorter compared to traditional Sanger methods, which prove to be a challenge when sequencing genes to characterize species.

Studies Using 454 Sequencing. Recent studies isolating bacteria from the feces of lamintic and healthy horses have relied on 454 sequencing for taxa identification (Steelman et al., 2012); however, little research exists targeting taxa in the equine cecum using these procedures. Steelman et al. (2012) concluded that *Firmicutes* was the most abundant phylum in both laminitic and healthy horses, accounting for 69.21% of the reported OTUs in healthy horses and 56.72% in horses suffering from chronic laminitis. The second most abundant phylum was *Verrucomicrobia* (18.13% healthy, 27.63% laminitis) followed by *Bacteriodets* (5.71% healthy, 9.94% lamintis). *Clostridiales*, *Lactobacilles,* and RFP12 of Verruco5 were the most abundant orders identified. On the family level*, Steptococcaceae* were the most dominant (24.17% healthy, 16.11% laminitis). The dominant genera were *Steptococcus* (21.00% healthy, 16.03% laminitis).

Other generas reported included, *Clostirdium, Treponema, Akkermansia, Oscillospira, Ruminococcus, Lactobacillus, Staphylococcus and Coprococcus*. Twelve of the twenty most abundant genera were unassigned, indicating a need for additional research in horses. Greater OTUs were detected compared to Sheperd et al. (2012) at a shorter read length due to the increase sampled size, indicating that any further research should be conducted with a sample size greater than two.

Statistical Analysis of Microbial Communities. Metagenomic analysis begins with assessing sequences based on quality and eliminating barcode sequences, primers, and noise (Barriuso at al., 2011). These steps are followed by aligning sequences, chimera detection, and filtering processes. Following these steps, OTUs are clustered to determine similarities and estimates of species richness using 3% and 5% similarity for species and genera assignments, respectively (Sun et al., 2009). Multiple software programs and algorithms have been developed for each of the analysis steps. Quantitative Insights into Microbial Ecology (QIIME) provides tools necessary for analysis and visualizations of taxa. QIIME is an open source software package that operates in a Unix based system and provides a way for users to visually depict their data and conduct statistical analysis on sequences (Caporaso et al., 2010). It allows for graphical depiction of histograms, OTU clusters, phylogenetic trees, and the α and β diversity of taxa in and between samples (Caporaso et al., 2010). The user can select scripts specific to the type of analysis required, and enter these commands into the terminal. QIIME can be used with other programs specific to individual analysis steps

including denoising sequences, chimera removal, or visually representing trends in datasets.

Quince et al. (2011) reported inflated estimates of OTUs before noise was removed from sequences. AmpliconNoise, DeNoiser, an SLP were compared on their ability to denoise sequences and construct OTU clusters. AmpliconNoise uses the PyroNoise and SeqNoise algorithms to cluster steps in a flowgram and align sequences. This algorithm is slower than DeNoiser, and SLP, but is superior in terms of per base error rates and OTU construction (Quince et al., 2011). Chimeric DNA sequences also form during PCR (Edgar et al., 2011). Chimeras form as a result of incomplete extension of the complimentary sequence. The incomplete sequence anneals to another parent strand in the next round of PCR (Edgar et al., 2011). Without the removal of erroneous chimeras, these sequences can be misjudged as novel species also leading to inflated OTU numbers. Studies indicate that datasets can contain up to 46% chimeras (Ashelford et al., 2005 and 2006; Huber et al., 2004). Chimera detection approaches include ChimeraSlayer (Haas et al., 2011), Perseus (Quince et al., 2011), and Decipher (Wright et al., 2012) and UCHIME (Edgar et al., 2011). ChimeraSlayer uses chimera-free reference sequences to construct alignments (Haas et al, 2011) while Perseus works best with sequences generated from 454 machines that have been subjected to AmpliconNoise beforehand (Quince et al., 2011). UCHIME shows greater sensitivity compared to other programs that require reference databases. Decipher and UCHIME provided the highest chimera detection for sequences 100-600 nucleotides long (Wright et al., 2012).

Diversity within and between samples will increase as the similarity in species structure decreases (Magurran, 2004). Statistical and phylogenetic analysis of microbial communities can be determined by analysis tools, such as QIIME. Species richness refers to the number of species in a given taxa in the specified assembly and will increase as more individuals are sampled (Magurran, 2004). If consistent sampling efforts are not accounted for, species richness fluctuates depending on site/location and size of sample due to individual variation (Gatson, 1996b).

Chao1 estimates the absolute number of species in a sample by comparing number of rare species to total number (Colwell and Coddington, 1994). Chao1 uses a ratio of singletons to doubletons to calculate species richness; thus, as the number of singletons increases in the sample so does species richness (Colwell and Coddington, 1994). However, singletons have been reported to be a product of pyrosequencing error, and must be eliminated prior to further analysis (Kunin et al., 2010; Tedersoo et al., 2010). In addition, Chao1 can overestimate species richness unlike ACE, which is a coverage estimator that offers greater precision (Chao et al., 2000). These estimators assume homogeneity amongst samples. Alpha (α) diversity refers to diversity in species observed at a particular site (Legendre et al., 2005). The α diversity metrics combine species richness and evenness to determine diversity (Magurran, 2004). The Shannon Index weights towards uncommon species and assumes all species are represented in a sample (Shannon, 1948). The Simpson Index takes into account dominance instead of richness and is weighted towards the most abundant species in a sample (Simpson, 1949). Despite the chosen index for measuring diversity, samples should be uniformly

rarefied to the lowest level of species richness found in a particular sample to eliminate bias. Rarefication ensures that species richness will not be overestimated (Fager, 1972). Beta (β) diversity refers to the variation between samples and reflects changes in species (Whitaker et al., 1972). Bray-Curtis Index uses dissimilarity and similiarity measures to calculate diversity. Morisita-Horn takes into account abundance of sequence to measure diversity (Chao, 2006). Following α and β diversity measurements, jackknife or bootstrap values can be used to assign confidence intervals to the statistic (Miller, 1974).

UniFrac, a phylogenetic diversity metric, detects differences in communities and tests if environmental factors are present (Lozupone and Knight, 2005). Environments can be compared using hierarchical clustering and principal coordinate analysis (PCA) (Krzanowski, 2000). UniFrac determines how divergent or similar microbial communities are based on the input from a phylogenetic tree. Simultaneous phylogenetic comparisons of different communities are attainable with the use of UniFrac.

Conclusions

Physiological and microbial responses to meal feeding have been thoroughly studied in ruminants; however, the horse has received little attention regarding specific changes in microflora in the gut. Alterations in microbial communities following addition of dietary starch can serve as a foundation for future research and may help to identify certain bacteria that may play a role in horses suffering from acute laminitis or other digestive disorders. Enumeration of microbes using pure culture (Kern et al 1973; 1974) and molecular techniques targeting the 16s rRNA gene using FISH, DGGE, and PCR (Lin and Stahl, 1995, Milinovich et al., 1995, Daly et al., 2001; Daly et al., 2011;

Onishi et al, 2012) have been investigated; however, little work has been done using 454 pyrosequencing to target 16s rRNA gene more accurately (Steelman et al., 2012). Therefore, the objective of this study is to determine changes in the complete community profile of microbiota in the equine cecum in response to abrupt dietary starch inclusion using 454 sequencing procedures.

CHAPTER II

MATERIALS AND METHODS

Care, handling, and sampling of animals were approved by the Texas A&M University Animal Care and Use Committee.

Horses and Dietary Treatments

Seven previously cecally cannulated Quarter horse geldings (10 to 23 yr; 497 to 580 kg) were used in a crossover design with two 28 d treatment periods separated by a 28 d wash out period between. The dataset reported in this thesis is a subset of a large scale study and focuses only on cecal samples taken during the first 12 h after concentrate meal exposure and the next 7 d of continued concentrate feeding. Prior to the start of the study, horses were paired based on BW and randomly assigned to one of two dietary treatments by pair. Horses received either 0.6% BW (LS; as fed basis) or 1.2% BW (HS; as fed basis) of commercial concentrate (Vitality Perform 14 Horse Feed; Cargill Animal Nutrition, Elk River, MN) fed individually twice daily at 0630 and 1830 (Table 1). The remainder of the diet consisted of ad libitum access to coastal bermudagrass hay (*Cynodon dactylon*) round bales group fed in a dry lot pen. The same hay was fed for 28 d prior to the start of period 1 and again during the wash out period. On d 0 of each period, BW was measured and the amount of concentrate offered was determined for the period. Horses were allowed 1 h to consume concentrate and refusals were recorded.

Table 1. Nutrient components of concentrate and forage (DM basis) fed to mature Quarter horse geldings

Item	Concentrate[1]	Forage[2]
DM, %	89.9	91.9
CP, %	14.0	9.6
NDF, %	14.3	70.1
ADF, %	5.9	36.2
NSC, %	30.0	---=
Ca, %	0.9	0.4
P, %	0.6	0.2

[1] Diets consisted of 0.06% BW = LS or 1.2% BW = HS (as fed) per day in commercially pelleted concentrate (Vitality Perform 14 Horse Feed; Cargill Animal Nutrition, Elk River, MN)
[2] Hay consisted of coastal bermudagrass (Cynodon dactylon) offered ad libitum

Sample Collection

All horses began treatments on d 1 of each period with no prior adaptation to determine the abrupt response to dietary concentrate. Cecal samples were collected prior to 0 h and 3, 6, 9, and 12 h after the morning meal on d 1 and 6 hr after the morning meal on d 2, 3, and 7 of each period. Cecal cannulas were opened and contents were collected into insulated containers. Immediately, pH was measured using a handheld pH meter (Thermo Orion, West Chester, PA). Approximately 30 ml of cecal contents were frozen at -20˚C for later isolation of DNA, and 10 mL was strained and frozen at -20˚C for later analysis of VFA. Samples intended for VFA analysis were processed using a 4:1 ratio of metaphosphoric acid:sample. All samples were then analyzed at Kansas State University using the procedure described by Vanzant and Cochran (1994)

DNA Isolation

Isolation of DNA from cecal samples for downstream identification analysis was performed in the Molecular Cytogenetics and Genomics Laboratory within the Texas

A&M University College of Veterinary Medicine. QIAamp DNA Mini Stool Kits (Qiagen, Valencia, CA) were utilized to insure rapid purification of DNA. Samples were vortexed until thawed in their vials and vortexed again for 5 min once thawed. Approximately 3 mL of cecal contents (particulate and fluid) were aliquoted into a 15 mL conical and suspended in 3 mL of Buffer ASL. All samples were kept on ice before suspension in Buffer ASL to prevent overgrowth of bacteria. Samples were centrifuged in 15 mL conical tubes at 500 rcf for 1 min and 500 µl of supernatant was immediately transferred into 2ml screw cap tubes containing 0.15 mm garnet beads (Mo Bio Laboratories, Inc., Carlsbad, CA). 1,000 µl of a 25 phenol: 24 chloroform: 1 isoamyl alcohol solution was added and vortexed at 1,500 rpm for 5 min. The suspension was incubated at 95˚C for 5 minutes and centrifuged to at 13,000 × g for 1 min to pellet stool particles. Immediately, 0.4 mL of supernatant was pipetted into a new 2 mL snap cap tube and suspended with 1 mL of ASL Buffer. One InhibitEX tablet was added to each sample and vortexed at 1,600 rpm for 5 min followed by incubation at room temperature for 1 min to allow inhibitors to absorb to the InhibitEX matrix. Samples were centrifuged at 16,000 × g for 7 min to pellet stool particles and inhibitors bound to the InhibitEX matrix. All supernatant was pipetted into a new 1.5 mL tube and centrifuged to pellet any remaining particles and inhibitors. 540 µl of supernatant was immediately transferred to a 2 mL screwcap tube containing 25 µl of Proteinase K. 540 µl of Buffer AL was added to the tube containing the supernatant and Proteinase K, vortexed for 15 s, and centrifuged for 5 s. Samples were incubated at 70˚C for 20 min and inverted several times. After incubation, samples were centrifuged to remove any droplets from the lid.

540 µl of 100% ethanol was added to the lysate, vortexed for 15 s, and centrifuged for 5 s. QIAamp spin columns were prepared and 540 µl of the ethanol/lysate was added to the column. Spin columns were centrifuged at 16,000 × g for 1 min. This step was repeated 3 times until the remainder or the ethanol/lysate was passed through the spin column. Spin columns were placed into new collection tubes after each centrifugation step. 500 µl of Buffer AW1 was added to the spin column and centrifuged at 16,000 × g for 1 min. 500µl Buffer AW2 was then added to the spin column and centrifuged at 16,000 × g for 4 min. Spin columns were then placed into new collection tubes and centrifuged again for 2 min at 16,000 × g to account for any residual carryover of Buffer AW2. Finally, 200 µl Buffer AE was pipetted directly on the spin column membrane. Samples incubated for 5 min at room temperature before centrifugation at 16,000 × g for 1.5 min in order to elute the DNA in a 0.5 mL screw cap tube. Before tubes were capped, 6 µl were aliquoted for gel electrophoresis and Nanodrop quantification. Samples were stored at -20˚C.

Eluted DNA was prepared for quality determination on 1% agarose gels. 100 mL of 0.5 TBE electrophoresis buffer was mixed thoroughly with 1.00g of agarose powder and warmed before adding 1µl ethidium bromide. Agar was poured in a gel tray with combs and allowed to cool before loading the gel. 3 µl of 2X orange loading dye was thoroughly mixed with 1µl of DNA and added to the wells of the gel. Approximately 3 µl of ladder and 1µl of 100bp standard were added to the gel for reference. Gels were inserted into an electrophoresis chamber, immersed in 0.5 TBE buffer, and diffused at 100 V. Following DNA migration in the electrophoresis chamber, gels were placed in a

ultraviolet illuminator to determine the size and integrity of DNA fragments. 2µl of the aliquot were Nano dropped to determine the quantity of the eluted DNA. All samples were then standardized to 20 ng/µl and diluted in water. Samples with quantities lower than 20 ng/µl were concentrated using sodium acetate and ethanol and re-suspended in Buffer AE.

454 Pyrosequencing

Pyrosequencing was performed with a Genome Sequencer FLX Titanium System (Roche, Branford, CT) at the MR DNA Molecular Research Lab (Shallowater, TX). The V4-V6 segment of the 16s rRNA gene was PCR amplified using the universal Eubacterial primer 530F (5'-GTGCCAGCMGCNGCGG-3') and 1100R (5'-GGGTTN CGNTCGTTG-3') and sequenced on the Roche 454 FLX platform according to the manufacturer's instructions (454 Life Sciences). Barcodes, primers, short sequences < 200 bp, and sequences with homopolymer runs exceeding 6 bp were removed. 890,204 sequences were denoised and chimera checked using a proprietary analysis pipeline (MR DNA, Shallowater, TX) prior to further analysis yielding a total of 670,371 sequences generating a mean of 7,288 reads per sample. Sequences were quality trimmed to 325 bp to ensure standardization after denoising and chimera checking.

Sequence Analysis

16s rRNA gene sequences were processed using QIIME (Caporaso et al., 2010). One sample was removed from analysis due to lack of sequence detection. Operational Taxonomic Units were classified based on a 97% sequences identity threshold using the UCLUST software package (Edgar, 2010). A representative sequence was selected for

each OTU based on the most abundant species. Sequences were aligned using PyNast (Caporaso et al., 2010) and taxonomy was assigned using BLAST against the latest Greenegenes database (http://greenegenes.lnl.gov/). GAPs were removed from sequences before tree construction using FastTree (Price et al., 2009). OTUs were considered part of the core microbiome if they were present in 100% of the samples in each treatment.

Statistical Analysis

Data were analyzed by repeated measures ANOVA using PROC MIXED procedure of SAS (SAS Inst. Inc., Cary, NC) with treatment, hour, day, and period in the model statement. This procedure was used to assess the main effects of period, treatment, time, and their interaction. Paired t-tests were used to test differences at each time point. Values of $P \leq 0.05$ were considered significant and $P \leq 0.10$ trended towards significance.

CHAPTER III

RESULTS

Cecal pH

Abrupt Response to Initial Concentrate Meal. Cecal pH tended to be influenced by treatment ($P \leq 0.09$; Figure 1) for the first 12 h after the first concentrate meal with HS horses having lower pH than LS horses. There was no effect of time or treatment by time interaction ($P \geq 0.29$). The decrease in cecal pH observed in HS horses at 3 h post meal is consistent with previous studies which reported that ingesta reached the cecum by 90 min post meal and decreased cecal pH by 4 h (Howell and Cupps, 1949). At 3 h post meal, HS and LS horses tended to be influenced by treatment ($P \leq 0.10$). It is important to note that at no time during this first 12 h after abrupt concentrate exposure did cecal pH reach levels indicative of subclinical acidosis which is defined as a pH less than 6 (Radicke et al., 1991). Following abrupt concentrate meal exposure, cecal pH of HS horses failed to return to baseline by 12 h post morning meal and start of evening meal, indicating a change in the bacterial community in the cecum.

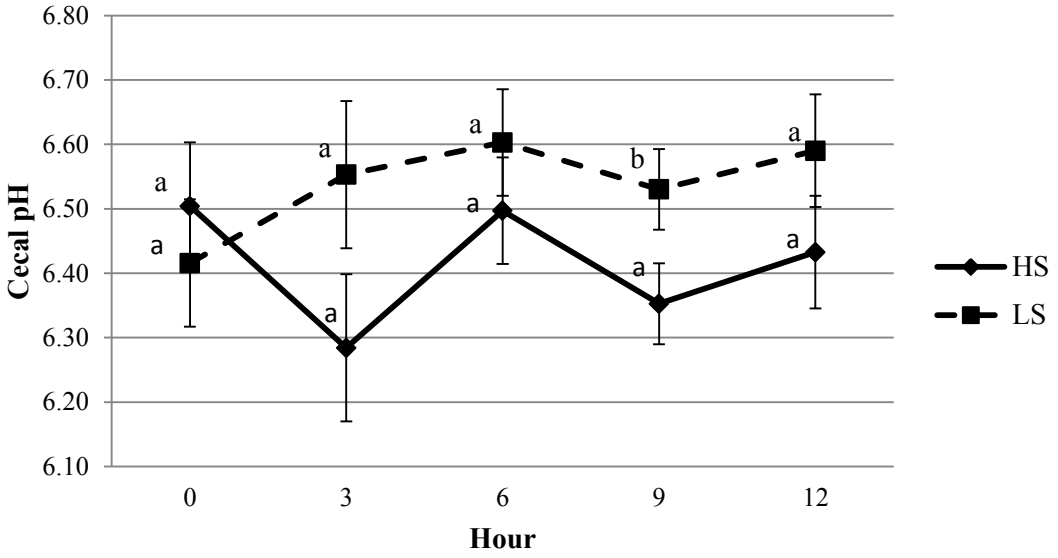

Figure 1. Influence of an abrupt concentrate diet adaptation (0.60% BW/d = LS; 1.20% BW/d = HS; as fed basis) on equine cecal pH prior to 0 h and 3, 6, 9, and 12 h after the initial meal. Values are LSMeans ± SE. Means with unlike superscripts significantly differ ($P \leq 0.05$) between treatments at each hour.

Continued Response Over 7 d. Over 7 d of concentrate feeding, HS horses had lower ($P \leq 0.05$; Figure 2) cecal pH at 6 h post morning meal compared to LS horses. Regardless of treatment, cecal pH was influenced by day ($P \leq 0.01$) with values decreasing on d 2 and 3 and failing to return to baseline by d 7. The cecal pH of both HS and LS horses followed similar patterns over 7 d, but HS horses had lower values at each measurement. Cecal pH was lowest for LS horses on d 2 and on d 3 for HS horses. Although pH measurements did not fall below 6 (subclinical acidosis), two HS horses displayed physical signs of lactic acidosis with body temperatures exceeding 38.8˚C on d 2 and 3 with pH values at or below 6. Values observed in LS horses at d 7 were similar to those recorded on d 1 ($P \geq 0.25$). However, HS horses had cecal pH values that were

lower ($P \leq 0.01$) on d 7 compared to d 1, indicating adaptation to concentrate meal feeding had not yet occurred.

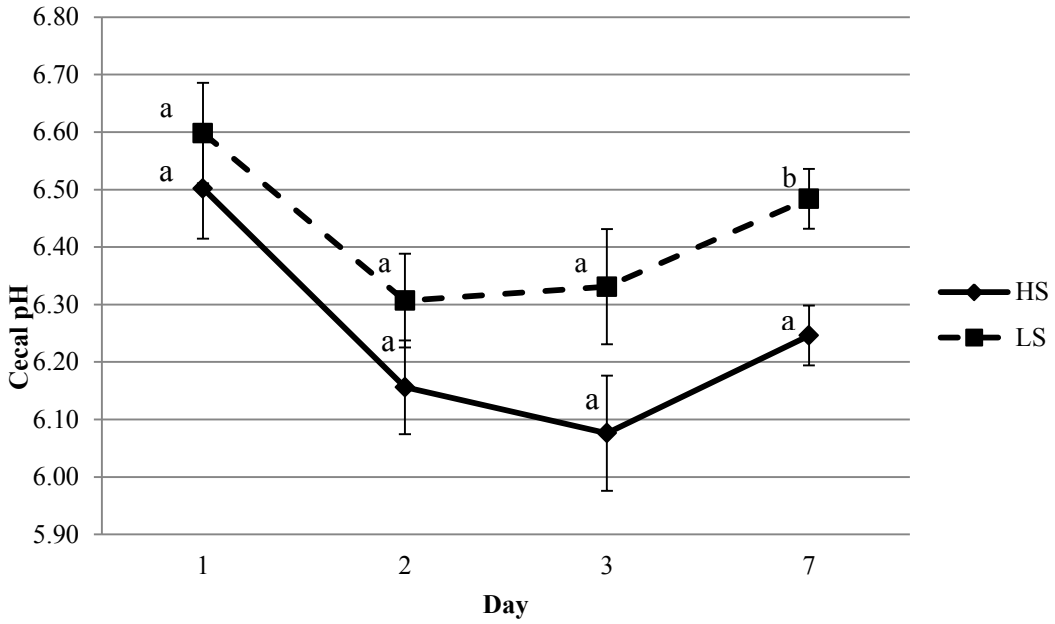

Figure 2. Influence of an abrupt concentrate diet adaptation (0.60% BW/d = LS; 1.20% BW/d = HS; as fed basis) on equine cecal pH obtained on d 1, 2, 3, and 7 at 6 h after the initial meal. Values are LSMeans ± SE. Means with unlike superscripts significantly differ ($P \leq 0.05$) between treatments at each hour.

Volatile Fatty Acids

Abrupt Response to Initial Concentrate Meal. Cecal VFA concentrations of acetate, propionate, butyrate, and valerate were influenced by treatment ($P \leq 0.05$; Table 2) in the first 12 h after abrupt concentrate meal administration with HS horses having higher concentrations compared to LS horses. Regardless of treatment, concentrations of acetate increased ($P \leq 0.03$) by 6 h post meal and then decreased to baseline at 12 h. A treatment by time interaction was observed with isovalerate ($P \leq 0.01$) concentrations

increasing from 0 to 3 h post meal in HS horses and increasing from 3 to 6 h post meal in LS horses.

Table 2. Volatile fatty acid concentration over first 12 h after an abrupt concentrate meal in the cecum of horses (represented as LSMeans)

	Diet[1]		SEM	*P*-Values[2]			
Item	HS	LS		Period	Trt	Hr	Trt x Hr
Acetate, *mM*	46.22	40.35	1.95	0.46	0.05	0.03	0.26
0 h	38.36	42.14					
3 h	52.20	39.51					
6 h	49.43	44.74					
9 h	47.84	40.28					
12 h	43.28	35.05					
Propionate, *mM*	16.33	13.39	0.70	0.75	0.01	0.15	0.08
0 h	11.78	13.51					
3 h	17.56	12.52					
6 h	16.40	14.60					
9 h	16.96	13.60					
12 h	18.97	12.70					
Butyrate, *mM*	4.90	4.15	0.24	0.26	0.04	0.69	0.26
0 h	4.08	4.56					
3 h	5.40	3.99					
6 h	5.01	4.50					
9 h	5.07	4.00					
12 h	4.97	3.63					
Valerate, mM	0.57	0.40	0.04	0.60	0.02	0.77	0.58
0 h	0.53	0.38					
3 h	0.58	0.33					
6 h	0.54	0.44					
9 h	0.57	0.42					
12 h	0.65	0.42					
Isobutyrate, *mM*	0.44	0.34	0.07	0.77	0.39	0.95	0.06
0 h	0.43	$0.32^{A,B,C}$					
3 h	0.48	0.28^{A}					
6 h	0.41	0.38^{B}					
9 h	0.45	$0.32^{A,C}$					
12 h	0.41	$0.40^{A,B,C}$					

Table 2. Continued

	Diet[1]		SEM	*P*-Values[2]			
Item	HS	LS		Period	Trt	Hr	Trt x Hr
Isovalerate, *mM*	0.38	0.30	0.07	0.37	0.44	0.09	0.01
0 h	0.31^{A}	0.23^{A}					
3 h	0.42^{B}	0.23^{A}					
6 h	$0.37^{A,B}$	0.34^{B}					
9 h	$0.42^{A,B}$	$0.29^{A,B}$					
12 h	$0.38^{A,B}$	0.39^{C}					

[1] Diets consisted of commercial concentrate (Vitality Perform 14 Horse Feed; Cargill Animal Nutrition, Elk River, MN) fed at 0.6% BW/d (LS) or 1.2% BW/d (HS; as fed basis). Remainder ad libitum access to coastal bermudagrass hay (*Cynodon dactylon*).
[2]Period = effect of period (1 and 2). Trt = effect of treatment (HS or LS). Hr = effect of time at 0, 3, 6, 9 and 12 h. Trt x Hr = interaction
[A-C] Values within a column lacking a common superscript differ by $P < 0.05$

Continued Response Over 7 d. In response to continued concentrate feeding over the 7 d, propionate concentrations were influenced by treatment ($P \leq 0.01$; Table 3) with HS horses having higher concentrations compared to LS. Concentrations of acetate decreased ($P < 0.08$) by d 2 and increased to reach baseline on d 7. Butyrate, valerate, and isovalerate were unaffected by treatment ($P \geq 0.13$). Regardless of treatment, valerate, isovalerate, and butyrate concentrations were influenced by d. Valerate concentrations decreased ($P \leq 0.02$) from d 1 to d 3 and returned to baseline by 7 d. Isovalerate and isobutyrate concentrations also decreased ($P \leq 0.02$ and $P \leq 0.02$) by d 2 in all horses and returned to baseline values by d 3 and decreased again at d 7. An effect of period was observed for acetate, propionate, and butyrate with decreased concentrations observed in period 2 compared to period 1 ($P \leq 0.01$, $P \leq 0.01$, $P \leq 0.05$, respectively), indicating the wash out between treatment periods may have been insufficient. Period effects may also be attributed to environmental factors such as

fluccuations in temperature and differences in hay nutrient content over the course of the study.

Table 3. Volatile fatty acid concentration over 7 d in the cecum of horses (represented as LSMeans)

	Diet[1]		SEM	*P*-Values[2]			
Item	HS	LS		Period	Trt	Day	Trt x Day
Acetate, *mM*	48.02	44.16	1.67	0.01	0.13	0.08	0.51
1 d	48.83	45.35					
2 d	44.53	42.93					
3 d	45.95	43.30					
7 d	52.78	45.08					
Propionate, *mM*	18.80	15.26	0.53	0.01	0.01	0.18	0.55
1 d	16.29	14.71					
2 d	21.23	15.99					
3 d	17.89	14.94					
7 d	19.79	15.41					
Butyrate, *mM*	4.74	4.50	0.23	0.05	0.48	0.82	0.80
1 d	5.00	4.52					
2 d	4.50	4.55					
3 d	4.75	4.62					
7 d	4.72	4.29					
Valerate, *mM*	0.47	0.44	0.03	0.90	0.43	0.02	0.39
1 d	0.55	0.44					
2 d	0.41	0.41					
3 d	0.40	0.42					
7 d	0.55	0.48					
Isobutyrate, *mM*	0.32	0.33	0.04	0.08	0.87	0.02	0.52
1 d	0.42	0.38					
2 d	0.25	0.28					
3 d	0.30	0.39					
7 d	0.32	0.28					

Table 3. Continued

	Diet[1]		SEM	*P*-Values[2]			
Item	HS	LS		Period	Trt	Day	Trt x Day
Isovalerate, *mM*	0.28	0.29	0.04	0.75	0.85	0.02	0.43
1 d	0.37	0.34					
2 d	0.18	0.23					
3 d	0.28	0.37					
7 d	0.28	0.23					

[1] Diets consisted of commercial concentrate (Vitality Perform 14 Horse Feed; Cargill Animal Nutrition, Elk River, MN) fed at 0.6% BW/d (LS) or 1.2% BW/d (HS; as fed basis). Remainder ad libitum access to coastal bermudagrass hay (*Cynodon dactylon*).
[2]Period = effect of period (1 and 2). Trt = effect of treatment (HS or LS). Day = effect of time at 1, 2, 3, and 7 d. Trt x Day = interaction

Microbial Populations

Taxonomic classification of cecal fluid identified 18 phyla, 35 classes, 70 orders, 120 families, and 208 genera. Unidentified genera were removed from the dataset as well as taxa that did not appear consistently enough in samples to allow statistical analysis. A total of 5,531 OTUs were observed with a minimum of 309 OTUs/sample and a maximum of 1,169 OTUs/sample. A total of 670,371 sequences were identified from all samples. A minimum of 1,612 sequences/sample and a maximum of 28,774 sequences/sample were observed. Figure 3 depicts the relationship of OTUs/sequences in each sample indicating that the number of OTUs/sample increases as more sequences were observed in each sample. As the number of sequences/sample approached 10,000, no additional OTUs could be detected. The majority of taxonomic variation and richness that 454 pyrosequencing can detect is accounted for when sequences/sample approaches10,000.

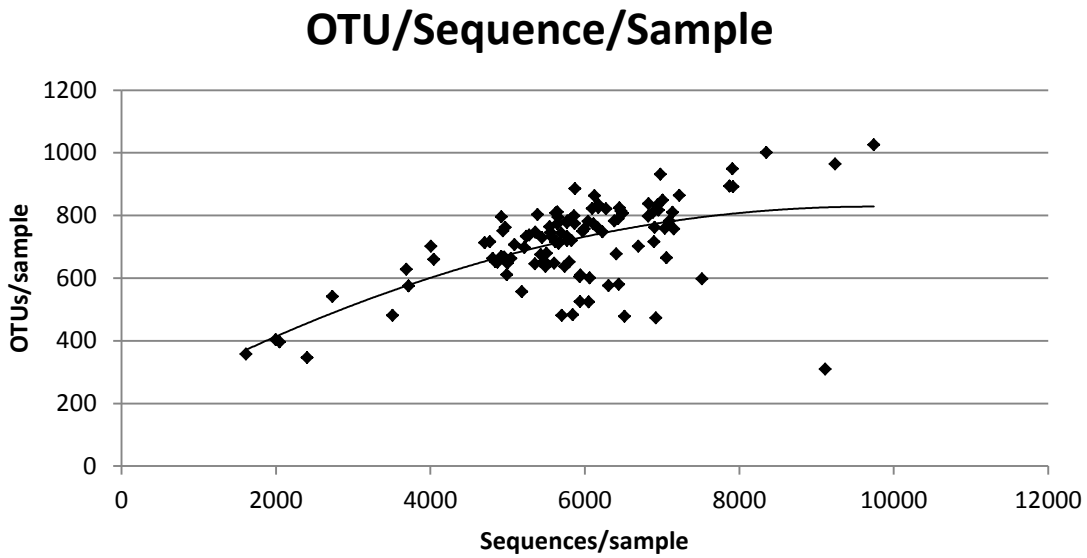

Figure 3. OTU/sequence present in each cecal sample following pyrosequencing and OTU classification taken from horses fed either 0.6% BW/d (LS) or 1.2% BW/d (HS; as fed basis) of an abrupt concentrate meal

Phylum

Approximately 18 observed phyla were identified, and the majority of sequences belonged to *Bacteriodetes* (69.82%) followed by *Firmicutes* (19.58%). The third most abundant phyla were *Proteobateria* (2.56%), followed by *Verrucomicrobiota* (2.27%), *Tenericutes* (1.14%), *Spirochaetes* (1.12%), and *Fibrobacteres* (1.18%). Phyla accounting for less than 1% of sequences included *Cyanobacteria, Fusobacteria, Synergistetes, TM7, WPS2, Plantomycetes, Elusimicrobia, Actinobcteria, Cloroflexi, Deferribacteres, and GN02*. Effects of period, treatment, time, and treatment x time interaction on phyla present in greater than 1% relative abundance are displayed over the first 12 h in Table 4 and over 7 d in Table 5. Complete tables of all phyla observed over

the first 12 h and over the next 7 days can be found in Table A1 and A2, respectively, in the Appendix.

Acute Response to Initial Concentrate Meal. Over the first 12 h after the initial concentrate meal, *Verrucomicrobiota* was influenced by treatment ($P \leq 0.04$) with LS horses having a higher relative abundance of the taxa than HS horses. *Spirochaetes* tended to be influenced by treatment ($P \leq 0.08$) with HS horses having a higher relative abundance compared to LS horses. Regardless of treatment within the first 12 h, relative abundances of *Bacteriodetes* increased ($P \leq 0.02$) at 9 h, while *Tenericutes* decreased ($P \leq 0.03$) from 2.62% to 1.91% by 12 h. Relative abundances of *Proteobacteria* decreased ($P \leq 0.01$) by 3 h post meal and increased to a percentage surpassing baseline by 12 h. The relative abundance of sequences assigned to *Fibrobacteres* also decreased ($P \leq 0.05$) over the 12 h post meal.

An effect of period was observed in the abrupt response to dietary starch over the first 12 h in *Bacteriodetes* ($P \leq 0.01$) and *Cyanobacteria* ($P \leq 0.04$) with increased relative abundances observed in period 2 compared to period 1. *Firmicutes* ($P \leq 0.01$) and Fusobacteria ($P \leq 0.03$) were influenced by period with decreased abundances observed in period 2 compared to period 1. Effects of period indicate a 28 d washout period may not have been adequate for microbial populations to return to baseline. A treatment by time interaction was observed for *Cyanobacteria* ($P \leq 0.03$) in HS horses with relative abundances increasing from 6 to 9 h while in LS horses relative abundances increased from 3 to 6 h.

Table 4. Percentage of sequences present in greater than 1% relative abundance assigned to phylum over first 12 h in the cecum of horses (LSMeans).

	Diet[1]		SEM	*P*-Values[2]			
Phylum	HS	LS		Period	Trt	Hr	Trt x Hr
Bacteriodetes, %	69.95	71.47	0.01	0.01	0.35	0.02	0.73
0 h	69.89	69.76					
3 h	68.60	70.79					
6 h	69.85	71.19					
9 h	71.94	73.43					
12 h	69.46	72.19					
Firmicutes. %	19.29	17.88	0.82	0.01	0.25	0.19	0.91
0 h	18.61	18.48					
3 h	21.02	19.41					
6 h	20.18	17.17					
9 h	17.01	16.69					
12 h	19.60	17.67					
Proteobacteria, %	1.96	1.20	0.40	0.19	0.20	0.01	0.20
0 h	2.27	1.18					
3 h	1.11	1.13					
6 h	2.27	0.92					
9 h	2.20	1.02					
12 h	1.96	1.75					
Verrucomicrobia, %	1.90	3.40	0.47	0.78	0.04	0.33	0.72
0 h	2.36	3.78					
3 h	2.50	2.74					
6 h	1.24	4.34					
9 h	1.16	2.90					
12 h	2.25	3.53					
Tenericutes, %	2.35	2.32	0.34	0.90	0.94	0.03	0.44
0 h	2.54	2.69					
3 h	2.75	2.64					
6 h	2.29	2.54					
9 h	2.05	2.02					
12 h	2.12	1.70					

Table 4. Continued

	Diet[1]		SEM	*P*-Values[2]			
Phylum	HS	LS		Period	Trt	Hr	Trt x Hr
Spirochaetes, %	2.73	1.96	0.28	0.92	0.08	0.77	0.54
0 h	2.29	2.00					
3 h	2.52	1.74					
6 h	2.33	2.20					
9 h	3.46	2.13					
12 h	3.05	1.75					
Fibrobacteres, %	1.46	1.28	0.23	0.10	0.60	0.05	0.82
0 h	1.63	1.56					
3 h	1.14	1.23					
6 h	1.51	1.18					
9 h	1.82	1.42					
12 h	1.16	1.00					

[1] Diets consisted of commercial concentrate (Vitality Perform 14 Horse Feed; Cargill Animal Nutrition, Elk River, MN) fed at 0.6% BW/d (LS) or 1.2% BW/d (HS; as fed basis). Remainder ad libitum access to coastal bermudagrass hay (*Cynodon dactylon*).
[2]Period = effect of period (1 and 2). Trt = effect of treatment (HS or LS). Hr = effect of time at 0, 3, 6, 9 and 12 h. Trt x Hr = interaction
[A-B] Values within a column lacking a common superscript differ by $P < 0.05$

Continued Response Over 7 d. In response to continued concentrate feeding over 7 d, *Verrucomicriobiota* and *Tenericutes* were influenced by treatment ($P \leq 0.02$ and $P \leq 0.02$) with LS horses having higher relative abundances compared to HS horses. *Proteobacteria* tended to be influenced by treatment ($P \leq 0.10$) with HS horses having a higher relative abundance of the taxa compared to LS. Regardless of treatment over 7 d, relative abundances of *Fibrobacteres* decreased ($P \leq 0.01$) from 1.35% on d 1 to 0.65% on d 7. However, relative abundances of *Firmicutes* increased ($P \leq 0.02$) steadily from 18.68% on d 1 to 22.78% on d 7 while *Tenericutes* decreased ($P \leq 0.01$) by d 2 and failing to return to baseline by d 7.

Period effects were also observed for *Bacteriodetes* ($P \leq 0.01$) and *Cyanobacteria* ($P \leq 0.01$) with increased relative abundances observed in period 2 compared to period 1. *Firmicutes* ($P \leq 0.01$) was also influenced by period with decreased abundances observed in period 2 compared to period 1.

Table 5. Percentage of sequences present in greater than 1% relative abundance assigned to phylum over 7 d in the cecum of horses (LSMeans).

	Diet[1]		SEM	*P*-Values[2]			
Phylum	HS	LS		Period	Trt	Day	Trt x Day
Bacteriodetes, %	67.30	70.53	1.60	0.01	0.18	0.66	0.51
1 d	70.11	70.93					
2 d	67.89	72.81					
3 d	61.95	69.26					
7 d	69.31	69.11					
Firmicutes, %	21.50	19.62	0.81	0.01	0.13	0.02	0.90
1 d	20.05	17.30					
2 d	19.72	18.97					
3 d	22.18	20.68					
7 d	24.03	21.52					
Proteobacteria, %	5.65	1.34	1.73	0.43	0.10	0.37	0.30
1 d	2.26	0.93					
2 d	6.28	1.19					
3 d	1.17	1.40					
7 d	2.37	1.83					
Verrucomicrobia, %	1.12	2.68	0.40	0.10	0.02	0.22	0.34
1 d	1.20	4.38					
2 d	1.44	2.27					
3 d	0.90	2.27					
7 d	0.93	1.78					
Tenericutes, %	1.58	2.33	0.21	0.20	0.02	0.01	0.02
1 d	2.31^{A}	2.52^{A}					
2 d	0.94^{B}	1.55^{B}					
3 d	1.38^{B}	2.81^{A}					
7 d	$1.67^{A,B}$	2.43^{A}					

Table 5. Continued

	Diet[1]		SEM	*P*-Values[2]			
Phylum	HS	LS		Period	Trt	Day	Trt x Day
Spirochaetes, %	1.74	1.98	0.25	0.31	0.51	0.15	0.28
1 d	2.35	2.18					
2 d	2.35	1.65					
3 d	1.26	2.02					
7 d	0.99	2.08					

[1] Diets consisted of commercial concentrate (Vitality Perform 14 Horse Feed; Cargill Animal Nutrition, Elk River, MN) fed at 0.6% BW/d (LS) or 1.2% BW/d (HS; as fed basis). Remainder ad libitum access to coastal bermudagrass hay (*Cynodon dactylon*).
[2]Period = effect of period (1 and 2). Trt = effect of treatment (HS or LS). Day = effect of time at 1, 2, 3, and 7 d. Trt x Day = interaction
[A-B] Values within a column lacking a common superscript differ by $P < 0.05$

Class

Approximately 35 classes of bacteria were identified following taxonomic classification. Main classes observed exceeding 1% abundance were *Bacteriodia* (69.44%), *Clostridia* (18.19%), *Mollicutes* (2.14%), *Spirochetes* (2.10%), *Gammaproteobacteria* (1.99%), *Verruco5* (1.58%), and *Fibrobacteria* (1.18%).

Order

Out of 70 orders of identified taxa, 7 were present at greater than 1% abundance. *Bacteriodales* (69.44%), *Clostridiales* (17.07%), *Spirochaetales* (2.03%), *WCHB141* (1.92%), *Pasteurellales* (1.58%), and *Fibrobacterales* (1.18%) were the dominant orders.

Family

Approximately 120 families were observed after taxonomic identification. UnknownFamily02 (27.59%), *Paraprevotellaceae* (19.68%), *Prevotellaceae* (15.92%),

Lachnospiraceae (8.85%), *Porphyromonadaceae* (4.84%), *Ruminococcaceae* (3.48%), *Veillonellaceae* (3.37%), *Spirochaetaceae* (2.03%), UnknownFamily31 (1.92%), *RFP12* (1.58%), *Pasteurellaceae* (1.27%), and *Fibrobacteraceae* (1.18%) were the 12 most dominant taxa. Family present in greater than 1% relative abundance are displayed over the first 12 h in Table 6 and over 7 d in Table 7. Complete tables of all family observed over the first 12 h and over the next 7 days can be found in Table A3 and A4, respectively, in the Appendix.

Abrupt Response to Initial Concentrate Meal. Over the first 12 h, *Paraprevotellaceae* was greater ($P \leq 0.01$) and *Spirochaetaceae* tended to be greater ($P \leq 0.08$) in HS horses having higher relative abundances compared to LS. The family *RFP12* was influenced and *Desulfovibrionaceae* tended to be influenced by treatment ($P \leq 0.05$ and $P \leq 0.08$) with LS horses in both families having higher relative abundances. S24-7 tended to be influenced by treatment ($P \leq 0.06$) with LS horses having a higher relative abundance compared to HS. Regardless of treatment, relative abundances of S24-7 in both HS and LS horses increased ($P \leq 0.03$) by 12 h.

Regardless of treatment within the first 12 h, abundances of *Prevotellaceae, Veillonellaceae*, *Streptococcaceae*, and *Succinivibrionaceae* increased ($P \leq 0.03$, $P \leq 0.01$, $P \leq 0.01$, $P \leq 0.01$, respectively) steadily. *Lachnospiraceae, Ruminococcaceae*, UnknownFamily31, UnknownFamily 10, and *Clostridiaceae* were also influenced by time with relative abundances decreasing ($P \leq 0.03$, $P \leq 0.03$, $P \leq 0.02$, $P \leq 0.01$, $P \leq 0.01$, respectively) steadily over 12 h. Relative abundances of *Porphyromonaceae* decreased ($P \leq 0.01$) at 6 h, peaked at 9 h, and decreased again at 12 h. Relative

abundances of *Fibrobacteraceae* decreased ($P \leq 0.05$) at 6 h, returned to baseline at 9 h, and decreased again by 12 h. *Verrucomicrobiaceae* increased ($P \leq 0.05$) at 3 h and returned to baseline by 12 h. Relative abundances of UnknownFamily09 peaked ($P \leq 0.03$) by 3 h and decreased past baseline by 12 h. A similar trend was observed in *Coprobacillaceae* with relative abundances increased ($P \leq 0.03$) at 6 h then declining past baseline at 12 h. Additionally, *Anaeroplasataceae* ($P \leq 0.01$) was influenced by time with relative abundances peaking at 9 h and decreasing past baseline at 12 h.

A period effect was observed for *Porphyromonadaceae, Anaeroplasmataceae,* and *Succinivibrionaceae* with greater ($P \leq 0.01$, $P \leq 0.03$, $P \leq 0.01$, respectively) abundances observed in period 2 compared to period 1. Decreased abundances of *Veillonellaceae, Clostridiaceae*, UnknownFamily10, *Copobacillaceae,* UnknownFamily04, and *Fusobacteria* were observed in period 2 compared to period 1 ($P \leq 0.04$, $P \leq 0.01$, $P \leq 0.01$, $P \leq 0.01$, $P \leq 0.04$, $P \leq 0.05$, respectively).

Table 6. Percentage of sequences present in greater than 1% relative abundance assigned to family over first 12 h in the cecum of horses (LSMeans).

	Diet[1]		SEM	*P*-Values[2]			
Family	HS	LS		Period	Trt	Hr	Trt x Hr
Unknown Family02, %	26.28	30.86	2.12	0.90	0.15	0.27	0.90
0 h	25.70	30.91					
3 h	26.83	33.42					
6 h	27.30	31.69					
9 h	27.45	31.30					
12 h	24.10	27.00					

Table 6. Continued

	Diet[1]		SEM	*P*-Values[2]			
Family	HS	LS		Period	Trt	Hr	Trt x Hr
Paraprevotellaceae, %	21.90	18.37	0.68	0.52	0.01	0.87	0.95
0 h	21.53	18.99					
3 h	23.04	19.02					
6 h	21.43	18.05					
9 h	21.65	17.58					
12 h	21.87	18.20					
Prevotellaceae, %	14.81	15.42	1.29	0.78	0.74	0.03	0.88
0 h	13.93	14.50					
3 h	13.56	13.15					
6 h	14.48	15.07					
9 h	15.54	16.10					
12 h	16.54	18.28					
Lachnospiraceae, %	9.10	8.73	0.64	0.27	0.64	0.03	0.95
0 h	9.90	9.48					
3 h	10.90	9.78					
6 h	9.38	8.50					
9 h	7.71	7.68					
12 h	7.91	8.23					
Porphyromonadaceae, %	4.73	5.41	0.43	0.01	0.29	0.01	0.35
0 h	6.12	4.54					
3 h	3.20	4.02					
6 h	4.05	5.14					
9 h	5.42	7.16					
12 h	4.87	6.21					
Ruminococcaceae, %	3.45	3.04	0.23	0.49	0.24	0.03	0.13
0 h	3.65	3.19					
3 h	4.37	3.26					
6 h	2.99	3.45					
9 h	3.06	2.82					
12 h	3.18	2.50					

Table 6. Continued

	Diet[1]		SEM	*P*-Values[2]			
Family	HS	LS		Period	Trt	Hr	Trt x Hr
Veillonellaceae, %	2.63	2.72	0.20	0.04	0.74	0.01	0.64
0 h	1.58	2.10					
3 h	1.97	2.42					
6 h	2.98	2.42					
9 h	2.95	3.02					
12 h	3.64	3.66					
Spirochaetaceae, %	2.61	1.88	0.27	0.99	0.08	0.80	0.54
0 h	2.18	1.91					
3 h	2.40	1.67					
6 h	2.24	2.11					
9 h	3.29	2.03					
12 h	2.93	1.67					
UnknownFamily31, %	2.18	2.03	0.34	0.94	0.77	0.02	0.25
0 h	2.30	2.42					
3 h	2.60	2.33					
6 h	2.15	2.27					
9 h	1.86	1.67					
12 h	1.97	1.47					
RFP12, %	1.21	2.70	0.49	0.78	0.05	0.57	0.78
0 h	1.66	3.01					
3 h	1.33	2.01					
6 h	0.70	3.54					
9 h	0.86	2.27					
12 h	1.53	2.66					
Fibrobacteraceae, %	1.46	1.28	0.23	0.10	0.60	0.05	0.82
0 h	1.63	1.56					
3 h	1.14	1.23					
6 h	1.51	1.18					
9 h	1.84	1.42					
12 h	1.16	1.00					

[1] Diets consisted of commercial concentrate (Vitality Perform 14 Horse Feed; Cargill Animal Nutrition, Elk River, MN) fed at 0.6% BW/d (LS) or 1.2% BW/d (HS; as fed basis). Remainder ad libitum access to coastal bermudagrass hay (*Cynodon dactylon*).
[2]Period = effect of period (1 and 2). Trt = effect of treatment (HS or LS). Hr = effect of time at 0, 3, 6, 9 and 12 h. Trt x Hr = interaction
[A-B] Values within a column lacking a common superscript differ by $P < 0.05$

Continued Response Over 7 d. Over 7 d, *Porphyromonadaceae* and *RFP12* were influenced by treatment ($P \leq 0.01$ and $P \leq 0.04$) with LS horses having a higher relative abundance of taxa compared to HS horses. In contrast, *Veillonellaceae* and *Succinivibrionaceae* increased ($P \leq 0.01$ and $P \leq 0.01$) in HS horses compared to LS. UnknownFamily02, UnknownFamily31, and *Aneroplasmaaceae* tended to be influenced by treatment ($P \leq 0.10$, $P \leq 0.06$, $P \leq 0.07$, respectively) with LS horses having a higher relative abundance compared to HS horses. Paraprevotellaceae tended to be influenced by treatment ($P \leq 0.07$) with HS horses having a greater relative abundance compared to LS horses.

Regardless of treatment over 7 d, the relative abundances of *Veillonellaceae* and *Erysipellotrichaceae* increased ($P \leq 0.01$ and $P \leq 0.04$) while *Fibrobacteraceae* decreased ($P \leq 0.01$). UnknownFamily31 was influenced by day with relative abundances decreasing ($P \leq 0.01$) by 2 d then increasing by 7 d, whereas relative abundances of *Succinivibrionaceae* increased ($P \leq 0.01$) by 3 d and declined and failed to return to baseline by 7 d. Relative abundances of *Desulfovibrionaceae* decreased ($P \leq 0.04$) on 2 d, increased by 3 d yet failed to return to baseline by 7 d.

A period effect was observed in *Porphyromonadaceae, Succinivibrionaceae*, and UnkownFamily04 with increased relative abundances observed in period 2 compared to period 1 ($P \leq 0.01$, $P \leq 0.01$, $P \leq 0.03$, respectively). Relative abundances of *RFP12, Pasteurellaceae, Clostridiaceae, Streptococcaceae, Lachnospiraceae,* UnknownFamily10*, Coprobacillaceae*, and *Erysipelotrichaceae* decreased ($P \leq 0.05$, *P*

≤ 0.05, $P \leq 0.01$, $P \leq 0.01$, $P \leq 0.01$, $P \leq 0.01$, $P \leq 0.02$, $P \leq 0.01$, respectively) in period 2 compared to period 1.

Table 7. Percentage of sequences present in greater than 1% relative abundance assigned to family over 7 d in the cecum of horses (LSMeans).

	Diet[1]		SEM	*P*-Values[2]			
Family	HS	LS		Period	Trt	D	Trt x D
UnknownFamily02, %	24.42	29.36	1.99	0.29	0.10	0.07	0.76
1 d	27.72	31.28					
2 d	22.43	29.06					
3 d	20.48	27.31					
7 d	27.04	29.80					
Paraprevotellaceae, %	20.51	17.71	0.99	0.35	0.07	0.24	0.93
1 d	21.48	18.00					
2 d	21.75	19.22					
3 d	19.37	17.67					
7 d	19.42	15.94					
Prevotellaceae, %	17.00	16.37	0.94	0.08	0.64	0.36	0.93
1 d	14.62	14.92					
2 d	17.87	16.78					
3 d	17.10	17.04					
7 d	18.42	16.73					
Lachnospiraceae, %	8.38	9.11	0.49	0.01	0.32	0.98	0.68
1 d	9.26	8.62					
2 d	8.38	9.10					
3 d	7.58	9.54					
7 d	8.31	9.19					
Porphyromonadaceae, %	3.53	5.46	0.49	0.01	0.01	0.09	0.85
1 d	4.03	5.16					
2 d	4.02	6.02					
3 d	3.44	5.88					
7 d	2.61	4.78					

Table 7. Continued

	Diet[1]		SEM	*P*-Values[2]			
Family	HS	LS		Period	Trt	D	Trt x D
Ruminococcaceae, %	3.50	3.90	0.41	0.32	0.50	0.12	0.98
1 d	2.97	3.47					
2 d	2.91	3.37					
3 d	3.61	3.83					
7 d	4.51	4.93					
Veillonellaceae, %	4.86	3.17	0.35	0.11	0.01	0.01	0.27
1 d	2.97	2.44					
2 d	4.61	3.12					
3 d	5.13	3.84					
7 d	6.75	3.29					
Spirochaetaceae, %	1.69	1.90	0.25	0.31	0.57	0.15	0.28
1 d	2.26	2.10					
2 d	2.31	1.58					
3 d	1.22	1.92					
7 d	0.96	2.00					
UnknownFamily31, %	1.43	2.05	0.21	0.36	0.06	0.01	0.01
1 d	2.16^{A}	2.25^{A}					
2 d	0.80^{B}	1.29^{B}					
3 d	1.21^{B}	2.52^{A}					
7 d	1.55^{A}	2.15^{A}					
RFP12, %	0.62	1.86	0.38	0.05	0.04	0.34	0.20
1 d	0.69	3.55					
2 d	0.75	1.51					
3 d	0.41	1.46					
7 d	0.63	0.94					
Pasteurellaceae, %	3.94	0.32	1.88	0.05	0.20	0.65	0.40
1 d	1.38	0.12					
2 d	4.18	0.19					
3 d	9.34	0.29					
7 d	0.84	0.68					

Table 7. Continued

	Diet[1]		SEM	*P*-Values[2]			
Family	HS	LS		Period	Trt	D	Trt x D
Fibrobacteraceae, %	0.91	1.08	0.15	0.15	0.46	0.01	0.13
1 d	1.49	1.20					
2 d	1.11	1.23					
3 d	0.52	1.09					
7 d	0.52	0.78					

[1] Diets consisted of commercial concentrate (Vitality Perform 14 Horse Feed; Cargill Animal Nutrition, Elk River, MN) fed at 0.6% BW/d (LS) or 1.2% BW/d (HS; as fed basis). Remainder ad libitum access to coastal bermudagrass hay (*Cynodon dactylon*).
[2]Period = effect of period (1 and 2). Trt = effect of treatment (HS or LS). Day = effect of time at 1, 2, 3, and 7 d. Trt x Day = interaction
[A-D] Values within a column lacking a common superscript differ by $P < 0.05$

Genus

Out of 208 genera, *Prevotella* (15.92%), *YRC22* (6.01%), *CF231* (5.96%), *Prevotella1* (3.42%), *Treponema* (2.03%), *Paludibacter*(1.93%), *Phascolarctobacterium* (1.83%), *Anaerovibrio* (1.47%), *Roseburia* (1.30%) and *Fibrobacter* (1.18%) were identified as the 10 known dominant genera exceeding 1% of sequences. Other identified generamaking up less than 1% of the sequences included *Streptococcus* (0.70%), *Akkermansia* (0.69%), *Coprococcus* (0.68%), *Ruminococcus* (0.39%), *Bacteroides* (0.38%), *Clostridium* (0.36%), *Oscillospira* (0.31%), *Ruminococcus* (0.31%), and *Desulfovibrio* (0.29%). Results for genera present in greater than 1% relative abundance are displayed over the first 12 h in Table 8 and over the next 7 d in Table 9. Complete tables of all genera observed over the first 12 h and over the next 7 days can be found in Table A5 and A6, respectively, in the Appendix.

Abrupt Response to Initial Concentrate Meal. Over the first 12 h, *Treponoma* tended to be influenced by treatment ($P \leq 0.08$) with HS horses having a higher relative abundance of the taxa compared to LS. *Desulfovibrio* was also influenced by treatment ($P \leq 0.08$) with LS horses having a higher relative abundance compared to HS horses. Regardless of treatment within the first 12 h, relative abundances of *Prevotella*, *Anaerovibrio*, and *Streptococcus* increased ($P \leq 0.03$, $P \leq 0.04$, $P \leq 0.01$, respectively) over the first 12 h. Relative abundances of *CF231*, *Coprococcus*, and *Ruminococcus* decreased ($P \leq 0.02$, $P \leq 0.01$, $P \leq 0.01$, respectively) over the first 12 h. *Paludibacter* ($P \leq 0.01$) was influenced by time with relative abundances decreasing and returning to baseline by 12 h. *Fibrobacter* ($P \leq 0.05$) was influenced by time with relative abundances decreasing, returning to baseline by 9 h, and decreasing again by 12 h. *Akkermansia* ($P \leq 0.05$) was influenced by time with relative abundances increasing by 9 h and returning to baseline by 12 h. *Clostridium* ($P \leq 0.01$) was influenced by time with relative abundances peaking at 3 h and declining past baseline at 12 h. *Oscillospira* ($P \leq 0.01$) was influenced by time with relative abundances increasing at 9 h and decreasing by 12 h. A period effect was observed in *Paludibacter* with increased abundances observed in period 2 compared to period 1 ($P \leq 0.01$). Decreased abundances in period 2 were reported *in Coprococcus*, *Ruminococcus*, and *Clostridium* ($P \leq 0.01$, $P \leq 0.03$, $P \leq 0.01$, respectively).

Table 8. Percentage of sequences present in greater than 1% relative abundance assigned to genus over first 12 h in the cecum of horses (LSMeans).

	Diet[1]		SEM	*P*-Values[2]			
Genus	HS	LS		Period	Trt	Hr	Trt x Hr
Prevotella, %	14.81	15.42	1.29	0.78	0.74	0.03	0.88
0 h	13.92	14.50					
3 h	13.56	13.15					
6 h	14.48	15.07					
9 h	15.54	16.10					
12 h	16.54	18.28					
YRC22, %	7.35	5.26	0.95	0.18	0.14	0.33	0.95
0 h	7.41	5.75					
3 h	9.05	6.31					
6 h	7.21	5.31					
9 h	6.35	4.25					
12 h	6.72	4.66					
CF231, %	6.33	6.56	0.77	0.74	0.83	0.02	0.34
0 h	6.39	7.77					
3 h	7.48	7.07					
6 h	5.93	6.54					
9 h	6.31	6.48					
12 h	5.53	4.99					
Prevotella1, %	4.02	3.03	0.84	0.82	0.41	0.69	0.86
0 h	3.99	2.33					
3 h	3.79	2.50					
6 h	3.55	2.69					
9 h	4.17	3.06					
12 h	4.82	4.57					
Treponema, %	2.61	1.88	0.27	0.99	0.08	0.80	0.54
0 h	2.18	1.91					
3 h	2.40	1.67					
6 h	2.24	2.11					
9 h	3.29	2.03					
12 h	2.93	1.67					

Table 8. Continued

	Diet[1]		SEM	*P*-Values[2]			
Genus	HS	LS		Period	Trt	Hr	Trt x Hr
Paludibacter, %	1.75	2.33	0.24	0.01	0.11	0.01	0.63
0 h	2.63	2.19					
3 h	0.87	1.47					
6 h	1.16	2.10					
9 h	2.15	3.23					
12 h	1.91	2.66					
Phascolarctobacterium, %	1.70	1.64	0.16	0.64	0.79	0.24	0.30
0 h	1.25	1.48					
3 h	1.61	1.75					
6 h	1.75	1.58					
9 h	1.88	1.87					
12 h	2.01	1.51					
Anaerovibrio,%	0.88	1.02	0.19	0.17	0.62	0.04	0.82
0 h	0.32	0.55					
3 h	0.33	0.61					
6 h	1.18	0.80					
9 h	1.03	1.06					
12 h	1.56	2.10					
Roseburia, %	1.34	1.40	0.30	0.90	0.88	0.56	0.33
0 h	1.06	1.44					
3 h	1.11	1.19					
6 h	2.04	1.09					
9 h	1.13	1.01					
12 h	1.35	2.28					
Fibrobacter, %	1.46	1.28	0.23	0.10	0.60	0.05	0.82
0 h	1.63	1.56					
3 h	1.14	1.23					
6 h	1.51	1.18					
9 h	1.84	1.42					
12 h	1.16	1.00					

[1] Diets consisted of commercial concentrate (Vitality Perform 14 Horse Feed; Cargill Animal Nutrition, Elk River, MN) fed at 0.6% BW/d (LS) or 1.2% BW/d (HS; as fed basis). Remainder ad libitum access to coastal bermudagrass hay (*Cynodon dactylon*).
[2]Period = effect of period (1 and 2). Trt = effect of treatment (HS or LS). Hr = effect of time at 0, 3, 6, 9 and 12 h. Trt x Hr = interaction

Continued Response Over 7 d. Over 7 d, *YRCC22* and *Anaerovibrio* were influenced by treatment ($P \leq 0.04$ and $P \leq 0.03$) with HS horses having higher relative abundances of the genus compared to LS. *Paludibacter* was influenced by treatment ($P \leq 0.01$) with LS horses having a higher relative abundance of the genus compared to HS.

Regardless of treatment, relative abundances of *Phascolarctobacterium* and *Anaerovibrio* increased ($P \leq 0.01$ and $P \leq 0.01$) by 7 d, whereas abundances of Fibrobacter decreased ($P \leq 0.01$). Relative abundances of *Desulfovibro* increased by 2 d ($P \leq 0.04$), then decreased and failed to return to baseline by 7 d.

A period effect was observed in *Paludibacter* with increased abundances observed in period 2 compared to period 1($P \leq 0.01$). Decreased abundances of *Anaerovibrio, Roseburia, Streptococcus, Ruminococcus,* and *Clostridium* were observed in period 2 compared to period 1 ($P \leq 0.10$, $P \leq 0.01$, $P \leq 0.1$, $P \leq 0.01$, $P \leq 0.05$, respectively).

Table 9. Percentage of sequences present in greater than 1% relative abundance assigned to genus over 7 d in the cecum of horses (LSMeans).

	Diet[1]		SEM	*P*-Values[2]			
Genus	HS	LS		Period	Trt	D	Trt x D
Prevotella, %	17.00	16.37	0.95	0.08	0.64	0.36	0.93
1 d	14.62	14.92					
2 d	17.87	16.78					
3 d	17.10	17.03					
7 d	18.42	16.73					
YRC22, %	6.82	4.55	0.70	0.38	0.04	0.74	0.56
1 d	7.16	5.36					
2 d	6.45	5.13					
3 d	6.41	3.86					
7 d	7.24	3.85					

Table 9. Continued

	Diet[1]		SEM	*P*-Values[2]			
Genus	HS	LS		Period	Trt	D	Trt x D
CF231, %	4.95	5.96	0.45	0.38	0.14	0.43	0.67
1 d	5.95	6.52					
2 d	4.76	6.15					
3 d	4.83	5.31					
7 d	4.27	5.88					
Prevotella1, %	3.67	3.74	0.44	0.74	0.17	0.26	0.36
1 d	3.35	2.69					
2 d	5.28	2.93					
3 d	3.23	3.12					
7 d	2.83	2.23					
Treponema, %	1.69	1.90	0.25	0.31	0.57	0.15	0.28
1 d	2.26	2.10					
2 d	2.31	1.58					
3 d	1.22	1.92					
7 d	0.96	2.00					
Paludibacter, %	1.15	2.28	0.17	0.01	0.01	0.34	0.75
1 d	1.15	2.12					
2 d	1.50	2.31					
3 d	1.10	2.49					
7 d	0.84	2.22					
Phascolarctobacterium, %	2.22	1.73	0.18	0.58	0.08	0.01	0.30
1 d	1.76	1.57					
2 d	1.92	1.70					
3 d	2.26	1.72					
7 d	2.93	1.91					
Anaerovibrio, %	2.54	1.40	0.32	0.01	0.03	0.01	0.47
1 d	1.11	0.86					
2 d	2.63	1.36					
3 d	2.75	2.09					
7 d	3.65	1.31					

Table 9. Continued

	Diet[1]		SEM	*P*-Values[2]			
Genus	HS	LS		Period	Trt	D	Trt x D
Roseburia, %	1.59	0.96	0.29	0.01	0.15	0.86	0.62
1 d	1.98	1.15					
2 d	1.89	0.91					
3 d	1.26	0.94					
7 d	1.25	0.86					
Fibrobacter, %	0.91	1.08	0.15	0.15	0.46	0.01	0.13
1 d	1.49	1.20					
2 d	1.11	1.23					
3 d	0.52	1.09					
7 d	0.52	0.78					

[1] Diets consisted of commercial concentrate (Vitality Perform 14 Horse Feed; Cargill Animal Nutrition, Elk River, MN) fed at 0.6% BW/d (LS) or 1.2% BW/d (HS; as fed basis). Remainder ad libitum access to coastal bermudagrass hay (*Cynodon dactylon*).
[2]Period = effect of period (1 and 2). Trt = effect of treatment (HS or LS). Day = effect of time at 1, 2, 3, and 7 d. Trt x Day = interaction
[A-B] Values within a column lacking a common superscript differ by $P < 0.05$

Core Microbiome

In order to be considered part of the core microbiome, OTUs were required to be present in 100% of the samples in each treatment. The core microbiome present in the sample taken before 0 h and prior to treatment administration consisted of 60 OTUS. *Bacteriodetes* accounted for the majority of OTUs (30), followed by *Firmicutes* (25), *Spirochaetes* (3), *Tenericutes* (1) and *Fibrobacteres* (1), respectively. Approximately 10 OTUs belonged to UnkownFamily02, UnkownFamily 31, UnkownFamily10, and UnknownFamily08, whereas 17 OTUs belonged to the *Lachnospiraceae* family. *Fibrobacter succinogenes*, the only sequence identified to species level, was observed to be present in 100% of the control samples. Table 10 displays the core microbiome across 100% of the control samples.

Table 10. Core microbiome across 100% of samples over 7 d in the cecum of horses prior to meal feeding at 0 h (control)[1]						
OTU #	Taxonomic Classification					
	Kingdom	Phylum	Class	Order	Family	Genus
2303	*Bacteria*	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*	*Prevotellaceae*	*Prevotella*
4420	*Bacteria*	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*	*Prevotellaceae*	*Prevotella*
6644	*Bacteria*	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*	*Prevotellaceae*	*Prevotella*
20	*Bacteria*	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*	*Prevotellaceae*	*Prevotella*
6320	*Bacteria*	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*	*Prevotellaceae*	*Prevotella*
3261	*Bacteria*	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*	*Prevotellaceae*	*Prevotella*
5579	*Bacteria*	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*	*Prevotellaceae*	*Prevotella*
6508	*Bacteria*	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*	*Prevotellaceae*	*Prevotella*
6878	*Bacteria*	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*	*Prevotellaceae*	*Prevotella*
5416	*Bacteria*	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*	*Paraprevotellaceae*	*Prevotella*
7076	*Bacteria*	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*	*Paraprevotellaceae*	*Prevotella*
5754	*Bacteria*	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*	*Paraprevotellaceae*	*CF231*
2631	*Bacteria*	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*	*Prevotellaceae*	
3814	*Bacteria*	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*	*Prevotellaceae*	
79	*Bacteria*	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*	*Prevotellaceae*	
2776	*Bacteria*	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*	*Prevotellaceae*	
35	*Bacteria*	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*		
6433	*Bacteria*	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*		
1794	*Bacteria*	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*		
7343	*Bacteria*	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*		
4486	*Bacteria*	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*		
5887	*Bacteria*	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*		
4960	*Bacteria*	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*		
2538	*Bacteria*	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*		
2889	*Bacteria*	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*		
82	*Bacteria*	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*		

Table 10. Continued						
OTU #	Taxonomic Classification					
	Kingdom	Phylum	Class	Order	Family	Genus
4217	*Bacteria*	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*	*Porphyromonadaceae*	
3126	*Bacteria*	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*	*Porphyromonadaceae*	
6532	*Bacteria*	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*	*Porphyromonadaceae*	*Paludibacter*
721	*Bacteria*	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*	*Porphyromonadaceae*	*Paludibacter*
808	*Bacteria*	*Firmicutes*	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	*Coprococcus*
1693	*Bacteria*	*Firmicutes*	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	*Coprococcus*
6034	*Bacteria*	*Firmicutes*	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	*Coprococcus*
3302	*Bacteria*	*Firmicutes*	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	*Lachnospira*
5933	*Bacteria*	*Firmicutes*	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	*Roseburia*
5899	*Bacteria*	*Firmicutes*	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	*Roseburia*
583	*Bacteria*	*Firmicutes*	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	
3566	*Bacteria*	*Firmicutes*	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	
7318	*Bacteria*	*Firmicutes*	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	
3573	*Bacteria*	*Firmicutes*	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	
3830	*Bacteria*	*Firmicutes*	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	
1125	*Bacteria*	*Firmicutes*	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	
5699	*Bacteria*	*Firmicutes*	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	
3179	*Bacteria*	*Firmicutes*	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	
4434	*Bacteria*	*Firmicutes*	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	
1729	*Bacteria*	*Firmicutes*	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	
2788	*Bacteria*	*Firmicutes*	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	
4186	*Bacteria*	*Firmicutes*	*Clostridia*	*Clostridiales*	*Ruminococcaceae*	
5927	*Bacteria*	*Firmicutes*	*Clostridia*	*Clostridiales*	*Peptococcaceae*	*rc4-4*
6091	*Bacteria*	*Firmicutes*	*Clostridia*	*Clostridiales*	*Clostridiaceae*	*Clostridium*
237	*Bacteria*	*Firmicutes*	*Clostridia*	*Clostridiales*	*Veillonellaceae*	*Phascolarctobacterium*
7151	*Bacteria*	*Firmicutes*	*Clostridia*	*Clostridiales*	*Veillonellaceae*	*Anaerovibrio*

Table 10. Continued						
OTU #	Taxonomic Classification					
	Kingdom	Phylum	Class	Order	Family	Genus
157	*Bacteria*	*Firmicutes*	*Erysipelotrichi*	*Erysipelotrichales*	*Coprobacillaceae*	
3027	*Bacteria*	*Firmicutes*	*Clostridia*	*Clostridiales*	*Ruminococcaceae*	
2130	*Bacteria*	*Spirochaetes*	*Spirochaetes*	*Spirochaetales*	*Spirochaetaceae*	*Treponema*
4742	*Bacteria*	*Spirochaetes*	*Spirochaetes*	*Spirochaetales*	*Spirochaetaceae*	*Treponema*
3499	*Bacteria*	*Spirochaetes*	*Spirochaetes*	*Spirochaetales*	*Spirochaetaceae*	*Treponema*
3911	*Bacteria*	*Firmicutes*	*Clostridia*	*Coriobacteriales*		
7195	*Bacteria*	*Tenericutes*	*Mollicutes*	*RF39*		
5988	*Bacteria*	*Fibrobacteres*	*Fibrobacteria*	*Fibrobacterales*	*Fibrobacteraceae*	*Fibrobacter*
[1] Ad libitum access to coastal bermudagrass hay (*Cynodon dactylon*).						

The core microbiome for LS horses was represented by 29 OTUs belonging to primarily the *Bacteriodetes* and *Firmicutes* phlym. An OTU belonging to the *Fibrobacteres*, *Verrucomicbrobia,* and *Spriochaetes* phyla was also observed. In the *Bacteriodetes* phylum, three unidentified genera were reported in the *Paraprevotellaceae* family, one unidentified genera was reported from the *Porphyromonadaceae* family, and five unidentified genera were reported in Unidentified Family 02. Two unidentified genera from each of the *Lachnospiraceae* and *Rumincococcaceae* family were reported across 100% of the samples in LS horses. One unknown genera was reported from the *Verrucomicrobia* phylum. Table 11 displays the core microbiome across 100% of the samples in LS horses.

Table 11. Core microbiome across 100% of samples over 7 d in the cecum of horses fed 0.60% BW/d (LS; as fed)[1]						
OTU #	Taxonomic Classification					
	Kingdom	Phylum	Class	Order	Family	Genus
20	*Bacteria*	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*	*Prevotellaceae*	*Prevotella*
3261	*Bacteria*	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*	*Prevotellaceae*	*Prevotella*
6508	*Bacteria*	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*	*Prevotellaceae*	*Prevotella*
2303	*Bacteria*	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*	*Prevotellaceae*	*Prevotella*
1772	*Bacteria*	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*	*Prevotellaceae*	*Prevotella*
6001	*Bacteria*	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*	*Prevotellaceae*	*Prevotella*
7076	*Bacteria*	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*	*Paraprevotellaceae*	*Prevotella*
79	*Bacteria*	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*	*Paraprevotellaceae*	
2631	*Bacteria*	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*	*Paraprevotellaceae*	
2776	*Bacteria*	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*	*Paraprevotellaceae*	
82	*Bacteria*	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*	UnknownFamily02	
4960	*Bacteria*	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*	UnknownFamily02	
5887	*Bacteria*	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*	UnknownFamily02	
4592	*Bacteria*	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*	UnknownFamily02	
2538	*Bacteria*	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*	UnknownFamily02	
721	*Bacteria*	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*	*Porphyromonadaceae*	*Paludibacter*
3126	*Bacteria*	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*	*Porphyromonadaceae*	
237	*Bacteria*	*Firmicutes*	*Clostridia*	*Clostridiales*	*Veillonellaceae*	*Phascolarctobacterium*
2014	*Bacteria*	*Firmicutes*	*Clostridia*	*Clostridiales*	*Veillonellaceae*	*Phascolarctobacterium*
7151	*Bacteria*	*Firmicutes*	*Clostridia*	*Clostridiales*	*Veillonellaceae*	*Anaerovibrio*
5899	*Bacteria*	*Firmicutes*	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	*Rosburia*
1693	*Bacteria*	*Firmicutes*	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	*Coprococcus*
1729	*Bacteria*	*Firmicutes*	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	
3179	*Bacteria*	*Firmicutes*	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	
4186	*Bacteria*	*Firmicutes*	*Clostridia*	*Clostridiales*	*Ruminococcaceae*	
400	*Bacteria*	*Firmicutes*	*Clostridia*	*Clostridiales*	*Ruminococcaceae*	

Table 11. Continued						
OTU #	Taxonomic Classification					
	Kingdom	Phylum	Class	Order	Family	Genus
5988	*Bacteria*	*Fibrobacteres*	*Fibrobacteria*	*Fibrobacterales*	*Fibrobacteraceae*	*Fibrobacter*
6626	*Bacteria*	*Verrucomicrobia*	*Verruco-5*	*WCHB1-41*	*RFP12*	
4742	*Bacteria*	*Spirochaetes*	*Spirochaetes*	*Spirochaetales*	*Spirochaetaceae*	*Treponema*
[1] Ad libitum access to coastal bermudagrass hay (*Cynodon dactylon*).						

The core microbiome for HS horses was represented by 16 OTUS consisting of primarily of the phyla, *Bacteroidetes* and *Firmicutes*. The core microbiome for HS horses contained the least number of OTUs as compared to LS horses and the samples utilized as a control. One unknown family belonging to the *Bacteroidetes* phyla was identified. Three additional unknown families belonging to the *Bacteroidetes* phyla, that were present in the core microbiome in the samples utilized as a control, were not observed in the HS diet. Primary families observed included *Prevotellaceae*, *Paraprevotellaceae, Lachnospiraceae, and Ruminococcaceae.* A total of 5 OTUs grouped with the the dominant sopecies, *Prevotella*. Table 12 displays the core microbiome across 100% of the samples in HS horses.

Table 12. Core microbiome across 100% of samples over 7 d in the cecum of horses fed 1.20% BW/d (HS; as fed)[1]						
OTU #	Taxonomic Classification					
	Kingdom	Phylum	Class	Order	Family	Genus
4960	*Bacteria*	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*	Unknown Family 02	
20	*Bacteria*	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*	*Prevotellaceae*	*Prevotella*
5579	*Bacteria*	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*	*Prevotellaceae*	*Prevotella*
3261	*Bacteria*	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*	*Prevotellaceae*	*Prevotella*
5922	*Bacteria*	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*	*Prevotellaceae*	*Prevotella*
4420	*Bacteria*	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*	*Prevotellaceae*	*Prevotella*
2631	*Bacteria*	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*	*Paraprevotellaceae*	
79	*Bacteria*	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*	*Paraprevotellaceae*	*YRCC22*
3008	*Bacteria*	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*	*Paraprevotellaceae*	*Prevotella*
7076	*Bacteria*	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*	*Paraprevotellaceae*	
4186	*Bacteria*	*Firmicutes*	*Clostridia*	*Clostridiales*	*Ruminococcaceae*	
400	*Bacteria*	*Firmicutes*	*Clostridia*	*Clostridiales*	*Ruminococcaceae*	*Coprococcus*
1693	*Bacteria*	*Firmicutes*	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	*Roseburia*
5899	*Bacteria*	*Firmicutes*	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	*Roseburia*
5933	*Bacteria*	*Firmicutes*	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	*Phascolarctobacterium*
237	*Bacteria*	*Firmicutes*	*Clostridia*	*Clostridiales*	*Veillonellaceae*	
[1] Ad libitum access to coastal bermudagrass hay (*Cynodon dactylon*).						

Bacterial Diversity

Sequences/sample ranged from 1,612 to 28,774 with a median value of 5,827. Samples were rarefied to a sampling depth of 4,700 on 3% dissimilarity before alpha diversity was computed using the Shannon, Simpson, and observed species indices. While a few studies have investigated diversity and richness of OTUs in equine fecal samples with low animal numbers (Shepherd et al., 2012; Steelman et al., 2012), higher bacterial diversity was observed in the present study based on cecal microbiomes.

Abrupt Response to Initial Concentrate Meal. Shannon, Simpson, and Chao1 indices revealed no difference between treatments in the first 12 h. Regardless of treatment, the Simpson index revealed an effect of time ($P \leq 0.01$) with diversity declining by 12 h. Observed OTUs tended to be influenced by time with values decreasing over 12 h. Although all four indices were not influenced by treatment in the first 12 h, values for LS horses were always higher than HS horses. Table 13 displays alpha diversity over the first 12 h.

Table 13. Alpha diversity over first 12 h in the cecum of horses fed an abrupt concentrate meal of varying levels with no adaptation (LSMeans)

	Diet[1]		SEM	*P*-Values[2]			
Metric	HS	LS		Period	Trt	Hr	Trt x Hr
Shannon	7.6058	7.6079	0.130	0.16	0.99	0.13	0.16
0 h	7.7406	7.7032					
3 h	7.5528	7.7079					
6 h	7.5703	7.6722					
9 h	7.6013	7.6495					
12 h	7.5642	7.3067					

Table 13. Continued

	Diet[1]		SEM	*P*-Values[2]			
Metric	HS	LS		Period	Trt	Hr	Trt x Hr
Simpson	0.9856	0.9853	0.002	0.02	0.92	0.02	0.38
0 h	0.9876	0.9872					
3 h	0.9822	0.9858					
6 h	0.9860	0.9849					
9 h	0.9865	0.9872					
12 h	0.9855	0.9813					
Chao1	1090.1	1101.4	39.16	0.32	0.84	0.28	0.54
0 h	1118.9	1132.8					
3 h	1101.5	1122.3					
6 h	1101.9	1152.7					
9 h	1051.2	1112.2					
12 h	1077.16	987.2					
Observed OTUs	679.01	675.48	26.34	0.64	0.92	0.09	0.65
0 h	711.65	701.15					
3 h	691.73	702.27					
6 h	675.73	695.04					
9 h	660.63	665.27					
12 h	655.31	613.66					

[1] Diets consisted of commercial concentrate (Vitality Perform 14 Horse Feed; Cargill Animal Nutrition, Elk River, MN) fed at 0.6% BW/d (LS) or 1.2% BW/d (HS; as fed basis). Remainder ad libitum access to coastal bermudagrass hay (*Cynodon dactylon*).
[2]Period = effect of period (1 and 2). Trt = effect of treatment (HS or LS). Hr = effect of time at 0, 3, 6, 9 and 12 h. Trt x Hr = interaction

Continued Response Over 7 d. Shannon, Simpson, and Chao1 indices revealed no difference between treatments over 7 d. However, observed species and Chao1 indices were influenced by time ($P \leq 0.01$ and $P \leq 0.01$) with values decreasing over the first twelve hours after the addition of dietary concentrate. Shannon index tended to be influenced by time ($P \leq 0.06$) with values decreasing over the first twelve hours. These results are consistent with research performed by Pitta et al. (2009) who observed greater

richness and diversity in the rumen of steers fed coastal bermudagrass compared to a wheat diet. Table 14 displays alpha diversity over 7 d.

Table 14. Alpha diversity over 7 d in the cecum of horses fed an abrupt concentrate meal with varying levels of concentrate with no adaptation (LSMeans).

	Diet[1]		SEM	*P*-Values[2]			
Metric	HS	LS		Period	Trt	D	Trt x D
Shannon	7.23	7.69	0.208	0.67	0.14	0.06	0.09
1 d	7.55	7.67					
2 d	7.35	7.66					
3 d	6.88	7.77					
7 d	7.14	7.67					
Simpson	0.9739	0.9877	0.008	0.51	0.28	0.69	0.11
1 d	0.9857	0.9848					
2 d	0.9834	0.9868					
3 d	0.9458	0.9914					
7 d	0.9808	0.9880					
Chao1	969.42	1076.93	48.27	0.31	0.14	0.01	0.62
1 d	1101.89	1146.97					
2 d	934.73	1057.63					
3 d	917.07	1069.50					
7 d	923.98	1033.60					
Observed OTUs	604.05	678.69	33.13	0.08	0.13	0.01	0.10
1 d	673.23	693.60					
2 d	608.53	662.91					
3 d	560.95	684.75					
7 d	573.52	673.48					

[1] Diets consisted of commercial concentrate (Vitality Perform 14 Horse Feed; Cargill Animal Nutrition, Elk River, MN) fed at 0.6% BW/d (LS) or 1.2% BW/d (HS; as fed basis). Remainder ad libitum access to coastal bermudagrass hay (*Cynodon dactylon*).
[2]Period = effect of period (1 and 2). Trt = effect of treatment (HS or LS). Day = effect of time at 1, 2, 3, and 7 d. Trt x Day = interaction

UNIFRAC Analysis

Beta diversity was analyzed at 6 h on d 7 for horses receiving the HS and LS treatments. Unweighted UNIFRAC analysis was utilized to determine divergence and similarity between treatments. Figure 4 displays three axes of a principal component analysis (PCA) plot.

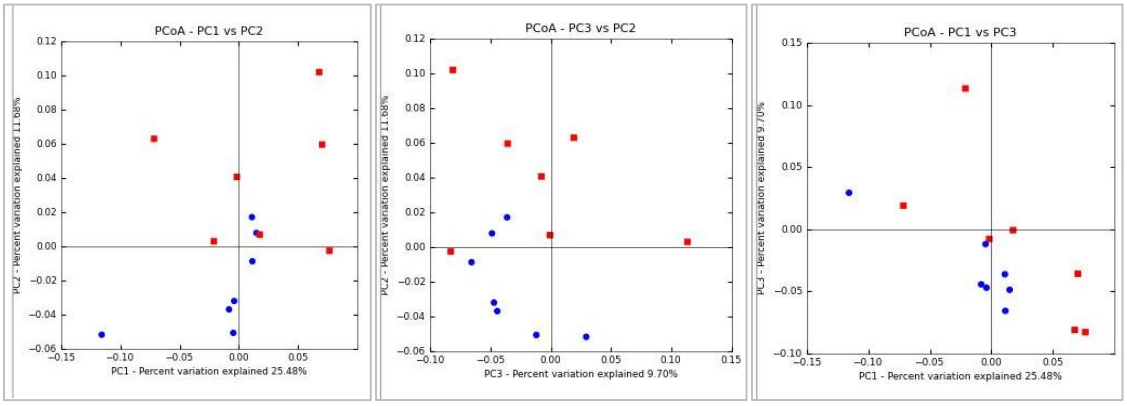

Figure 4. Principal coordinate analysis of weighted UniFrac distance between dietary treatment of either 0.6% BW/d (LS) or 1.2% BW/d (HS; as fed basis). a) PC1 vs PC2 b) PC3 vs PC2 c) PC1 vs PC3. Treatments indicated by color; red (LS) and blue (HS).

CHAPTER IV

DISCUSSION

During adaptation to dietary concentrate, horses are subjected to changes in cecal environment, specifically fermentative mechanisms of microflora that result in altered VFA ratios and changes in pH. Feeding regimens utilized by the performance horse industry do not provide sufficient time to fully adapt the microbial community to the change in substrate. These feeding strategies are often utilized when young horses are removed from pasture and immediately subjected to confinement and meal feeding that accompanies training regimens. Microbiome dynamics are poorly understood during abrupt and long-term meal feeding of concentrate in the horse, specifically the incorporation of NSC.

Diets used in the present study were not designed to cause starch overload resulting in gastrointestinal upset, acidosis, or laminitis. The majority of studies investigating the equine cecal environment have utilized animals subjected to euthanasia due to gastrointestinal or metabolic disease, or have targeted the feces with hopes of uncovering information related to cecal environment. Other studies utilized small sample sizes of animals and lack of time effects after meal feeding in their experimental design. Limited data using cannulated animals has hindered the ability to directly investigate factors directly affecting cecal environment, specifically pH, VFAs, and microbial populations, simultaneously at short-term abrupt or longer multi-day intervals.

Many taxa on the phylum, family, and genus level were affected by period within the first 12 h and over the next 7 days of continued concentrate feeding. This dataset was part of a large scale study in which samples were taken until d 28 with a 28 day washout between both periods, indicating this washout period may be insufficient. Other factors that may contribute to period effects are variation between hay, as roundbales were fed over the four month duration of this study. Increasing temperatures in summer months may also have contributed to period effects observed in the present study. A more thorough evaluation of the duration of a washout period needs to be investigated, as adaptation by microbes to substrate may continue past this timepoint.

Little is known about phenotypic characteristics of many taxa detected in the present study. Specifically, an unknown family belonging to the *Bacteriodetes* phylum accounted for an average of 25.35% of relative abundance of sequences in HS horses and 30.11% of relative sequences in LS horses. This was the dominant family with the largest percentage of sequences assigned, but little characteristics are known about this family as it has yet to be identified. A more thorough understanding of bacterial physiology and metabolomics may provide insight as to how microorganisms are directly influenced by dietary substrate. Although 16S rRNA sequencing can provide a snapshot of who exists in the community microbiome, little is known about unculturable microorgamisms and their functionality. Some genera of bacteria have been cultured in the past, providing some information about their metabolism and end products, but this only provides a partial representation of the fermentative mechanisms of the microbial community. In addition, more sensitive methods of microbial detection that surpass

capabilities of pyrosequencing are needed as sequence depth increases. The link between microbes and their metabolites has not been sufficiently studied, particularly in the horse, and the current study provides the framework for further investigation.

Cecal pH

Subclinical lactic acidosis in horses has been observed at cecal pH below 6 (Radicke et al., 1991); however, horses in the current study did not reach subclinical acidosis. An increase in lactic acid bacteria has also been suggested as a contributing factor to lactic acidosis in ruminants (Cotta et al., 1988) and horses (Al Jassim et al., 1999 and 2005). Julliand et al. (2001) concluded that cecal pH decreased as greater amounts of fermentable substrate are incorporated into the diet. Cecal pH of horses fed 100% hay decreased from 6.74 to 6.41 when 30% barley was added to the diet, and continued to decrease to 6.26 as 50% barley was added (Julliand et al., 2001). Although barley was fed instead of a commercial pellet, cecal pH from the present study followed a similar trend with HS horses having lower cecal pH at all time points compared to LS horses. The sharp decline in cecal pH in HS horses by 3 h may be related to increased production of lactic acid from starch fermenting bacteria that have yet to be identified.

Lactobacillus, a member of the *Firmicutes* phylum, was observed in the present study, but was unable to be statistically analyzed due to low coverage. This genus did not play a major role leading to a decline in cecal pH alone, but may contributed to the decline in pH when coupled with other lactic acid producing taxa. Although this genus has been shown to grow readily on selective agar in pure culture, agar media supply a concentrated substrate for the spp to grow unlike the competitive environment in the

cecum. In addition, *Lactobacillus* spp enumerated from horses administered oligofructose which provided a highly concentrated supply of nutrients for the species to proliferate (Milinovich, 2005). However, diets in the present study provided starch as a substrate instead of concentrated form of oligofructose. *Lactobacillus* abundance contributed to a decline in cecal pH; although not as pronounced in other studies inducing laminitis as a model, the genera still contributed to the decline in pH. Other *Firmicutes* genera observed in the present study have been reported as major lactic acid producers in the human and bovine gastrointestinal tract included, *Aerococcus, Gamella, Enterococcus, Lactobacillus, Weissella, Lactococcus, Turicibacter, Trichococcus, Marinilactibacillus, Facklamia,* and *Alloiococcus* (Kagki et al., 2007; Rattanachaikunsopon and Phumkhachorn, 2010). While these genera were present in samples from the current study, they were unable to be statistically analyzed for treatment, time, or period effects due to low coverage. Although these lactic acid producers wereof low abundance, together they would have contributed to a decline in pH. These observations support the need for more sensitive and accurate detection methods for analysis of changes across treatments and over time.

Members of the *Veillonellaceae* family ferment starch to produce lactate or ferment lactate to produce propionate and acetate which explains the increase in the taxa over the first 12 h with the addition of dietary starch regardless of treatment (Linehan, 1997). Relative abundances of *Veillonellaceae were* also higher in HS horses and increased over 7 d. *Selenomas, Phascolarctobacterium*, and *Veillonella* were present in low quantities. An increase of abundances at the family level suggests that its members,

specifically *Anaerovibrio*, have the ability to ferment lactate produced by lactic acid producing species. *Streptococcus,* a lactate producing species reported in the present study, did increase over the first 12 h with the addition of dietary starch. Mackie and Health, (1979) also observed an increase in amlyolytic bacteria accompanied by an increase in lactate fermenting bacteria as additional concentrate was fed to steers. This suggests a symbiotic relationship excisits between lactate producing and fermenting bacteria.

Volatile Fatty Acids

End products of fermentation have been studied to determine large scale changes in cellulolytic and amylolytic classes of bacteria; however, specific VFA production of the majority of the taxa identified in this study, especially those that have not been cultured previously, has not been evaluated. Propionate concentrations in the present study at 0 h were similar to horses maintained on grass-legume hay (Willard et al., 1977) and alfalfa/bromegrass hay (Coverdale et al., 2004). There were no differences between treatments for the concentration of acetate and butyrate in the first 12 h; however, propionate concentrations were numerically higher in HS horses. Julliand et al. (2001) observed an increase in both acetate and propionate concentrations, 5 h and 29 h post meal, when a diet was changed from 100% hay to 50% hay and 50% barley. A similar trend was observed in the present study regardless of treatment, where acetate concentrations increased by 6 h post-meal, indicating that soluble carbohydrates are actively fermented in the cecum 5 to 6 h post concentrate feeding. An increase in isovalerate was observed from 3 to 6 h post meal and 9 to 12 h post meal in LS horses.

Similarly, isovalerate concentrations also increased from 0 to 3 h in HS horses. Julliand et al. (2001) observed an increase in isovalerate from 5 h to 29 h; however, differences in diet composition between studies may lead to differences in fermentation end products and how quickly substrates are fermented. Abrupt influx of a highly fermentable diet modifies cecal VFA concentrations over the first 12 h.

Altering VFA ratios with the inclusion of rapidly fermentable CHO in the cecum of cannulated horses was also observed by Coverdale et al. (2004). Although soyhulls were fed instead of a commercial concentrate in the present study, cecal propionate concentrations increased with addition of a more highly fermentable CHO source, and values were similar to those reported in the present study at 6 h post meal. Therefore, incorporating greater amounts of highly fermentable substrate into the diet alters VFA ratios, indicating a change in microbial activity in the cecum.

Modifying the diet with graded amounts of dietary starch has been performed in the same herd of cannulated horses as used in the present study (Wilson, 2009). During their 28 d adaptation to dietary treatment, increasing levels of the amount of dietary starch resulted in increased cecal propionate, acetate, and buyrate concentrations. This is comparable to the increases observed in the current study. Wilson (2009) observed increased concentrations of propionate as increasing amounts (2, 4, 6, or 8 g/kg BW/d) of starch were fed. Although dietary treatments were fed for a longer period of time, 28 d vs. 7 d in the current study, similar trends were observed. A complete adaptation period needs to be thoroughly investigated as acetate, propionate, and butyrate were

influenced by period, indicating an insufficient wash out period between treatment periods.

Microbial Populations

Previous studies that identify microbes present in the cecum have used culture or molecular based techniques to determine quantities and abundances of bacteria. Little data exists that provides concurrent evidence of physiological changes of pH, VFAs, and microbes in the cecum of horses in response to meal feeding. Significant effects on the phylum level of taxa reported in the present study provide a starting point to investigate their roles in starch fermentation and homeostasis in the cecum on the family and genus level.

Bacteriodetes and *Firmicutes* have been reported to be the predominant phyla in the rumen (Fernando et al., 2010). Although molecular based tools were used to detect changes in community composition compared to 454 pyrosequencing used in the present study, 219 clones belonged to the *Firmicutes* phylum while 147 belonged to the *Bacteriodetes* phylum in steers fed prairie hay. Steers fed the high concentrate diet had an increase in clones belonging to the *Firmicutes* phylum (approximately 271) and an increase in clones belonging to the *Bacteriodetes* phylum (approximately 303). Similar trends were seen in horses as concentrate was added to the ration. In our study, *Bacteriodetes* increased steadily over 12 h regardless of treatment, and over 7 d, relative abundances of *Firmicutes* increased regardless of treatment. The increase in relative abundances of *Firmicutes* and *Bacteriodetes* in both steers and horses fed a concentrate diet indicates members of the phyla have the ability to ferment rapidly available

carbohydrates. This statement is supported by the fact that some members of the Firmicutes phylum, specifically members of the *Lactobacillales* and *Clostridiales*, are known lactic acid producers and utilizers, respectively (Makarova, 2006; Reddy, 2008). Although *Lactobacillus* did not have an effect of treatment or time, the presence of this taxa was still reported in a low relative abundance.

During the abrupt response phase to initial concentrate meal, higher abundances of *Paraprevotellaceae* were found in HS horses, and *Prevotellaceae* increased over time regardless of treatment. Members of these families have been shown to increase in abundance with the addition of a step up diet in ruminants until a level of 80% concentrate was added to the diet, indicating a rapid population growth of these taxa (Fernando et al., 2010).

Unknown Family 02 accounted for the majority (27.59%) of *Bacteriodetes* sequences abundance. Studies in ruminants have also concluded many species belonging to this phylum have yet to be accounted for, which suggests a large number of bacteria in the equine cecum have yet to be described (Fernando et al., 2010).

Proteobacteria also increased in abundance when steers were fed a step up diet with increasing amounts of starch, which suggests their ability to utilize rapidly fermentable carbohydrates (Fernando et al., 2010). Abundances of *Proteobacteria* were higher in HS horses than LS horses both in the first 12 h and over the next 7 d, indicating their role in starch fermentation. *Succinivibrionaceae*, a family of the *Proteobacteria* phylum, increased in the first 12 h regardless of treatment and relative abundances of the taxa were higher in HS horses over the 7 d period. Members of this family are known to

ferment carbohydrates and produce acetate and succinate, which could support the increased acetate production observed over the first 12 h.

Members of the *Spirochaete* phylum including the *Spirochetes* genus have been identified in the rumen (Stanton and Canale-Parola, 1979). Members of this phylum ferment pectin and starch and do not break down cellulose (Paster and Canale-Parola, 1981). Furthermore, *Spirochetes* can utilize glucose and cellobiose produced from rumen cellulolytic bacteria fermentation (Paster and Canale-Parola, 1981). The increase in their relative abundance in the HS diet may result from their ability to ferment starch. *Treponoma*, a non-cellulolytic member of the *Spirochaetes* phylum, ferments polysaccharides such as starch and pectin (Paster and Canale-Parola, 1981; Trkov et al., 2000). Sugar tests performed on pure culture indicate that *Treponoma* produces acetate, formate, and ethanol as end products of fermentation (Trkov et al., 2000). Acetate production from this genus could have contributed to the initial increase in acetate in the HS diet in the first 12 h. Members of the *Spirochaete* phylum are metabolically diverse in their ability to ferment NSC in the cecum.

Research has shown that some members of the *Verrucomicrobia* phylum play a role in cellulose breakdown. *Verrucomicrobia* has also been isolated from the feces of horses (Sheperd et al., 2012; Steelman et al., 2012). The HS diet in the present study may have inhibited these taxa from efficiently utilizing both structural CHO from the hay and nonstructural CHO from the grain, resulting in LS horses have higher relative abundances compared to HS. Members of this phylum oxidize methane and use it as a carbon and energy source deeming the taxa as methanotrophs (Dunfield et al., 2007).

They utilize end products of fermentation including acetate and H_2 coupled with CO_2 to produce methane. These bacteria may thrive under conditions that favor methane compared to high starch enriched diets. Members of this phylum also have the potential to produce propionate, which in turn can serve as a precursor for acetate production. This phylum is metabolically diverse in the production and utilization of metabolites and end products of fermentation, suggesting the LS diet in the present study supported taxa of this phylum's metabolic needs. Additionally, members of the *Verrucomicrobia* could be inhibited by an environment dominated by starch fermenting and lactic acid producing bacteria, which would explain the lower abundance of these taxa in the HS diet both in the first 12 h and over 7 d specifically in *RFP12,* which is a good candidate for culture. Strictly cellulolytic taxa have been reported to decline upon the addition of dietary starch into the diet.

The *Fibrobacteres* contains the cellulolytic genus, *Fibrobacter*, which ferments the cellulose component of plants. The expected decline in *Fibrobacteres* both in the first 12 h and over 7 d following introduction of dietary concentrate resulted from an increase in sequences from competitive starch fermenting species, specifically those belonging to the *Firmicutes* phylum. Changes over time observed for *Fibrobacteres* indicate members of the phyla may be still adapting to fermentable starch introduced in the diet. As the addition of starch continued over 7 d, competition between bacteria allowed starch fermenting species to increase and shift community composition. Cellulolytic genera including *Buyrivibrio* have been isolated from the rumen and equine cecum (Berger et al., 1990; Daly et al., 2001). Although, this genus was observed in the

present study, due to low coverage it was unable to be statistically analyzed for main effects. Similar trends were found in *Clostridium*, a member of the Firmicutes phylum, as relative abundances decreased over 12 h following the addition of dietary starch. Species in the *Clostridium* genus have cellulolytic activity and synthesize ceullulolytic enzyme complexes in the presence of cellulose in pure culture (Mayer et al., 1987). As cellulolytic spp decrease, microflora becomes altered, which changes the native microbiome of grazing horses and increases the risk of digestive upsets. Additionally, at certain thresholds, many microbes detected in the current study play a role in health of performance horse in order to utilize substrate to provide additional energy for the horse. If the microbiome becomes altered due to the inclusion of high amounts of NSC, negative effects on gastrointestinal health have been reported (Garner et al; 1977; Radicke et al.,1991; Milinovich et al., 2005, Costa and Weese, 2012). Other major phyla, specifically *Verrucomicrobia* and *Tenericutes,* had greater relative abudances in LS horses compared to HS horses in the first 12 h, or relative abundances that decreased over the first 12 h, respectively, indicating that surivival in a starch rich environment in less favorable. Additionally, these phyla may have some role in cellulose fermentation.

Gastrointestinal Health and Disease

The equine microbiome plays a crucial role in intestinal health and disease (Costa and Weese, 2012); therefore, understanding interactions between microorgansims and their host is important. Although laminitis is a multifactorial disease, one genus, *Streptococcus*, has been reported to play a causal role in the events prior to the onset of abrupt laminitis in horses (Garner et al., 1977; Milinovich et al., 2005). *Streptococcus* is

an amylolytic genus that has been reported to proliferate upon the addition of dietary starch and has been identified as a contributing factor associated with laminitis in horses, especially in the first 12 h after administration of oligofructose to induce laminitis (Milinovich et al., 2005). Relative abundances of *Streptococcus* increased over time regardless of dietary treatment in the current study, suggesting a response to addition of dietary NSC. This also indicates a role in starch fermentation and gastrointestinal health.

Core Microbiome

The core microbiome of HS horses consisted of members of the *Firmicutes* and *Bacteriodes* phylum, while the core microbiome of LS horses consisted of members of the *Bacteriodetes, Firmicutes, Fibrobacteres*, and *Spirochaetes* phylum. The presence of greater amounts taxa in LS horses and the greatest amount of taxa in the control samples are consistent with greater amounts dietary fiber that supports a more metabolically diverse and rich cecal microbiome, comprised of both starch and cellulose fermenters. Five different OTU's belonging to Unknown Family 02 in the *Bacteriodetes* phylum were present across 100% of the samples in the LS diet; however, little is known about the phylogeny of the family. Additional culture based techniques will be necessary to determine these taxa's role in the equine hindgut.

A greater number of OTUs were observed in samples taken at 0h consisting of strictly hay fed diets compared to the LS and HS diet, which is similar to research performed in steers transitioned from a bermudagrass diet to a grazed wheat diet (Pitta et al., 2010). A greater number of genera were observed in the hay diet compared to the wheat diet, indicating that diversity and richness of taxa also increase as more OTUs are

observed. Primary cellulolytic genera observed in the hay samples included, *Fibrobacter* and *Clostridum.* Members of the *Ruminococcaceae* were also observed in 100% of the samples utilized as a control at 0 h. A large number of OTUs were identified belonging to 4 unknown bacterial families, indicating more research is needed to culture and classify these bacteria. Additionally, 10 unknown generas belonging to the *Lachnospiraceae* family would make good candidates for culture and and classification.

Bacterial Diversity

The diversity estimates of the equine microbiome in response to dietary starch inclusion have yet to be adequately described. Studies in ruminants adapted to high grain diets have reported higher diversity and richness measures in hay-fed animals compared to concentrate-fed animals (Fernando et al., 2010). Higher Chao1 and ACE estimates were reported in animals consuming solely forage based diets compared to high grain diets (Fernando et al., 2010). Pitta et al. (2010) also observed greater diversity and richness in the rumen of steers fed coastal bermudagrass compared to a wheat diet. Although no treatment effects were observed between HS and LS horses in the present study, LS horses supported numerically greater diversity metrics compared to HS. Observed species were higher in LS horses compared to HS horses, which is consistent with studies performed in ruminants (Pitta et al., 2009; Fernando et al., 2010).

Unifrac distance relies on pylogenetic comparisons of diversity. Weighted UniFrac uses an algorithm to calculate specific weights of the branches of a phylogenetic tree. Weighted UniFrac Analysis revealed variation between HS and LS horses at the 6 h

timepoint on d 7. A clustering effect of HS horses revealed a similar community structure compared to LS horses which had more divergence between samples.

Conclusion

This study reports the physiological and microbial responses to meal feeding of performance horses. Results from this study can be used for investigating the role of specific microbes that are affected by the addition of dietary starch and their contribution to the equine microbiome in response to abrupt and long term adaptation to concentrate diets. Competition of bacteria in response to NSC inclusion modifies cecal environment by decreasing pH, and altering VFA concentrations and relative abundances of microbes. Higher bacterial diversity was present in the LS diet and additionally in the core microbiome of horses fed the LS diet, which may indicate a more favorable environment for cecal microbes. Additionally, apha and beta diversity analysis revealed a narrowing of the microbial community structure with the addition of dietary starch. This was further supported by core microbiome analysis, which revealed a narrowing of community structure specifically in the HS diet compared to the LS diet. Significant changes were reported in the phylum, family, and genus level community structure, as many taxa were affected by treatment and time in the first 12 h and over the next 7 d. Although our diets were fed below the threshold needed to induce starch overload or subclinical acidosis, diets in the present study with less than 1.8 gNSC/kg BW/meal do modify cecal environment, but not as extreme as those inducing laminitis or acidosis as a model.

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APPENDIX

Table A1. Percentage of sequences assigned to phylum over first 12 h in the cecum of horses (LSMeans).

	Diet[1]		SEM	*P*-Values[2]			
Phylum	HS	LS		Period	Trt	Hr	Trt x Hr
Bacteriodetes, %	69.95	71.47	0.01	0.01	0.35	0.02	0.73
0 h	69.89	69.76					
3 h	68.60	70.79					
6 h	69.85	71.19					
9 h	71.94	73.43					
12 h	69.46	72.19					
Firmicutes. %	19.29	17.88	0.82	0.01	0.25	0.19	0.91
0 h	18.61	18.48					
3 h	21.02	19.41					
6 h	20.18	17.17					
9 h	17.01	16.69					
12 h	19.60	17.67					
Proteobacteria, %	1.96	1.20	0.40	0.19	0.20	0.01	0.20
0 h	2.27	1.18					
3 h	1.11	1.13					
6 h	2.27	0.92					
9 h	2.20	1.02					
12 h	1.96	1.75					
Verrucomicrobia, %	1.90	3.40	0.47	0.78	0.04	0.33	0.72
0 h	2.36	3.78					
3 h	2.50	2.74					
6 h	1.24	4.34					
9 h	1.16	2.90					
12 h	2.25	3.53					
Tenericutes, %	2.35	2.32	0.34	0.90	0.94	0.03	0.44
0 h	2.54	2.69					
3 h	2.75	2.64					
6 h	2.29	2.54					
9 h	2.05	2.02					
12 h	2.12	1.70					

Table A1. Continued

	Diet[1]		SEM	*P*-Values[2]			
Phylum	HS	LS		Period	Trt	Hr	Trt x Hr
Spirochaetes, %	2.73	1.96	0.28	0.92	0.08	0.77	0.54
0 h	2.29	2.00					
3 h	2.52	1.74					
6 h	2.33	2.20					
9 h	3.46	2.13					
12 h	3.05	1.75					
Fibrobacteres, %	1.46	1.28	0.23	0.10	0.60	0.05	0.82
0 h	1.63	1.56					
3 h	1.14	1.23					
6 h	1.51	1.18					
9 h	1.82	1.42					
12 h	1.16	1.00					
Cyanobacteria, %	0.17	0.10	0.03	0.04	0.85	0.60	0.03
0 h	$0.15^{A,B}$	$0.16^{A,B}$					
3 h	$0.17^{A,B}$	0.11^{A}					
6 h	0.11^{A}	0.23^{B}					
9 h	0.22^{B}	$0.20^{A,B}$					
12 h	$0.17^{A,B}$	$0.17^{A,B}$					
Fusobacteria, %	0.09	0.03	0.06	0.03	0.49	0.45	0.36
0 h	0.10	0.02					
3 h	0.06	0.02					
6 h	0.04	0.03					
9 h	0.08	0.01					
12 h	0.16	0.07					

[1] Diets consisted of commercial concentrate (Vitality Perform 14 Horse Feed; Cargill Animal Nutrition, Elk River, MN) fed at 0.6% BW/d (LS) or 1.2% BW/d (HS; as fed basis). Remainder ad libitum access to coastal bermudagrass hay (*Cynodon dactylon*).
[2]Period = effect of period (1 and 2). Trt = effect of treatment (HS or LS). Hr = effect of time at 0, 3, 6, 9 and 12 h. Trt x Hr = interaction
[A-B] Values within a column lacking a common superscript differ by $P < 0.05$

Table A2. Percentage of sequences assigned to phylum over 7 d in the cecum of horses (LSMeans).

	Diet[1]		SEM	*P*-Values[2]			
Phylum	HS	LS		Period	Trt	Day	Trt x Day
Bacteriodetes, %	67.30	70.53	1.60	0.01	0.18	0.66	0.51
1 d	70.11	70.93					
2 d	67.89	72.81					
3 d	61.95	69.26					
7 d	69.31	69.11					
Firmicutes, %	21.50	19.62	0.81	0.01	0.13	0.02	0.90
1 d	20.05	17.30					
2 d	19.72	18.97					
3 d	22.18	20.68					
7 d	24.03	21.52					
Proteobacteria, %	5.65	1.34	1.73	0.43	0.10	0.37	0.30
1 d	2.26	0.93					
2 d	6.28	1.19					
3 d	1.17	1.40					
7 d	2.37	1.83					
Verrucomicrobia, %	1.12	2.68	0.40	0.10	0.02	0.22	0.34
1 d	1.20	4.38					
2 d	1.44	2.27					
3 d	0.90	2.27					
7 d	0.93	1.78					
Tenericutes, %	1.58	2.33	0.21	0.20	0.02	0.01	0.02
1 d	2.31^{A}	2.52^{A}					
2 d	0.94^{B}	1.55^{B}					
3 d	1.38^{B}	2.81^{A}					
7 d	$1.67^{A,B}$	2.43^{A}					
Spirochaetes, %	1.74	1.98	0.25	0.31	0.51	0.15	0.28
1 d	2.35	2.18					
2 d	2.35	1.65					
3 d	1.26	2.02					
7 d	0.99	2.08					
Fibrobacteres, %	0.91	0.10	0.15	0.15	0.46	0.01	0.13
1 d	1.49	1.20					
2 d	1.11	1.23					
3 d	0.52	1.09					

Table A2. Continued

	Diet[1]		SEM	*P*-Values[2]			
Phylum	HS	LS		Period	Trt	Day	Trt x Day
7 d	0.52	0.78					
Cyanobacteria, %	0.11	0.17	0.03	0.03	0.25	0.15	0.01
1 d	0.11	$0.23^{A,C}$					
2 d	0.13	0.11^{B}					
3 d	0.10	$0.21^{A,C}$					
7 d	0.11	$0.13^{A,B}$					

[1] Diets consisted of commercial concentrate (Vitality Perform 14 Horse Feed; Cargill Animal Nutrition, Elk River, MN) fed at 0.6% BW/d (LS) or 1.2% BW/d (HS; as fed basis). Remainder ad libitum access to coastal bermudagrass hay (*Cynodon dactylon*).
[2]Period = effect of period (1 and 2). Trt = effect of treatment (HS or LS). Day = effect of time at 1, 2, 3, and 7 d. Trt x Day = interaction
[A-B] Values within a column lacking a common superscript differ by $P < 0.05$

Table A3. Percentage of sequences assigned to family over first 12 h in the cecum of horses (LSMeans).

	Diet[1]		SEM	*P*-Values[2]			
Family	HS	LS		Period	Trt	Hr	Trt x Hr
Unknown Family02, %	26.28	30.86	2.12	0.90	0.15	0.27	0.90
0 h	25.70	30.91					
3 h	26.83	33.42					
6 h	27.30	31.69					
9 h	27.45	31.30					
12 h	24.10	27.00					
Paraprevotellaceae, %	21.90	18.37	0.68	0.52	0.01	0.87	0.95
0 h	21.53	18.99					
3 h	23.04	19.02					
6 h	21.43	18.05					
9 h	21.65	17.58					
12 h	21.87	18.20					
Prevotellaceae, %	14.81	15.42	1.29	0.78	0.74	0.03	0.88
0 h	13.93	14.50					
3 h	13.56	13.15					
6 h	14.48	15.07					
9 h	15.54	16.10					
12 h	16.54	18.28					
Lachnospiraceae, %	9.10	8.73	0.64	0.27	0.64	0.03	0.95
0 h	9.90	9.48					
3 h	10.90	9.78					
6 h	9.38	8.50					
9 h	7.71	7.68					
12 h	7.91	8.23					
Porphyromonadaceae, %	4.73	5.41	0.43	0.01	0.29	0.01	0.35
0 h	6.12	4.54					
3 h	3.20	4.02					
6 h	4.05	5.14					
9 h	5.42	7.16					
12 h	4.87	6.21					
Ruminococcaceae, %	3.45	3.04	0.23	0.49	0.24	0.03	0.13
0 h	3.65	3.19					

Table A3. Continued

	Diet[1]		SEM	*P*-Values[2]			
Family	HS	LS		Period	Trt	Hr	Trt x Hr
3 h	4.37	3.26					
6 h	2.99	3.45					
9 h	3.06	2.82					
12 h	3.18	2.50					
Veillonellaceae, %	2.63	2.72	0.20	0.04	0.74	0.01	0.64
0 h	1.58	2.10					
3 h	1.97	2.42					
6 h	2.98	2.42					
9 h	2.95	3.02					
12 h	3.64	3.66					
Spirochaetaceae, %	2.61	1.88	0.27	0.99	0.08	0.80	0.54
0 h	2.18	1.91					
3 h	2.40	1.67					
6 h	2.24	2.11					
9 h	3.29	2.03					
12 h	2.93	1.67					
UnknownFamily31, %	2.18	2.03	0.34	0.94	0.77	0.02	0.25
0 h	2.30	2.42					
3 h	2.60	2.33					
6 h	2.15	2.27					
9 h	1.86	1.67					
12 h	1.97	1.47					
RFP12, %	1.21	2.70	0.49	0.78	0.05	0.57	0.78
0 h	1.66	3.01					
3 h	1.33	2.01					
6 h	0.70	3.54					
9 h	0.86	2.27					
12 h	1.53	2.66					
Pasteurellaceae, %	0.75	0.17	0.41	0.08	0.34	0.16	0.39
0 h	0.25	0.02					
3 h	0.30	0.04					
6 h	1.41	0.09					
9 h	1.16	0.17					
12 h	0.63	0.52					

Table A3. Continued

	Diet[1]		SEM	*P*-Values[2]			
Family	HS	LS		Period	Trt	Hr	Trt x Hr
Fibrobacteraceae, %	1.46	1.28	0.23	0.10	0.60	0.05	0.82
0 h	1.63	1.56					
3 h	1.14	1.23					
6 h	1.51	1.18					
9 h	1.84	1.42					
12 h	1.16	1.00					
S247, %	0.50	0.99	0.16	0.38	0.06	0.03	0.46
0 h	0.46	0.74					
3 h	0.58	0.95					
6 h	0.41	0.96					
9 h	0.46	0.97					
12 h	0.60	1.31					
Clostridiaceae, %	0.75	0.76	0.06	0.01	0.91	0.01	0.92
0 h	0.80	0.96					
3 h	1.01	0.97					
6 h	0.64	0.62					
9 h	0.65	0.64					
12 h	0.65	0.60					
Streptococcaceae, %	0.87	0.32	0.24	0.40	0.13	0.01	0.40
0 h	0.13	0.08					
3 h	0.13	0.17					
6 h	0.14	0.11					
9 h	0.82	0.60					
12 h	0.18	0.66					
Verrucomicrobiaceae, %	0.64	0.77	0.30	0.51	0.78	0.05	0.67
0 h	0.66	0.68					
3 h	1.13	0.76					
6 h	0.50	0.84					
9 h	0.26	0.66					
12 h	0.68	0.90					
UnknownFamily10, %	0.67	0.60	0.05	0.01	0.31	0.01	0.08
0 h	0.78	0.81					
3 h	0.84	0.73					

Table A3. Continued

	Diet[1]		SEM	*P*-Values[2]			
Family	HS	LS		Period	Trt	Hr	Trt x Hr
6 h	0.70	0.57					
9 h	0.42	0.46					
12 h	0.60	0.41					
Succinivibrionaceae, %	0.37	0.24	0.08	0.01	0.31	0.01	0.18
0 h	0.19	0.13					
3 h	0.05	0.12					
6 h	0.22	0.16					
9 h	0.56	0.27					
12 h	0.83	0.53					
UnknownFamily09, %	0.48	0.51	0.07	0.26	0.78	0.03	0.47
0 h	0.62	0.64					
3 h	0.60	0.85					
6 h	0.39	0.44					
9 h	0.41	0.30					
12 h	0.36	0.32					
Bacteroidaceae, %	0.71	0.34	0.37	0.69	0.49	0.36	0.22
0 h	1.03	0.13					
3 h	0.56	0.18					
6 h	0.45	0.20					
9 h	0.76	0.22					
12 h	0.76	0.97					
UnknownFamily08, %	0.45	0.40	0.10	0.33	0.75	0.86	0.85
0 h	0.38	0.39					
3 h	0.39	0.38					
6 h	0.46	0.33					
9 h	0.41	0.42					
12 h	0.61	0.50					
Flavobacteriaceae, %	0.51	0.11	0.20	0.07	0.19	0.23	0.49
0 h	0.48	0.03					
3 h	0.31	0.08					
6 h	1.25	0.12					
9 h	0.19	0.13					
12 h	0.32	0.20					
Desulfovibrionaceae, %	0.23	0.41	0.06	0.84	0.08	0.22	0.31

Table A3. Continued

	Diet[1]		SEM	*P*-Values[2]			
Family	HS	LS		Period	Trt	Hr	Trt x Hr
0 h	0.22	0.56					
3 h	0.35	0.48					
6 h	0.21	0.44					
9 h	0.19	0.29					
12 h	0.19	0.31					
Coprobacillaceae, %	0.25	0.20	0.06	0.01	0.59	0.07	0.52
0 h	0.19	0.22					
3 h	0.32	0.24					
6 h	0.55	0.16					
9 h	0.13	0.21					
12 h	0.04	0.16					
UnknownFamily04, %	0.17	0.18	0.03	0.04	0.84	0.60	0.02
0 h	$0.15^{A,B}$	$0.16^{A,B}$					
3 h	$0.17^{A,B}$	0.11^{A}					
6 h	0.11^{A}	0.23^{B}					
9 h	0.22^{B}	$0.20^{A,B}$					
12 h	$0.17^{A.B}$	$0.17^{A.B}$					
Fusobacteriaceae, %	0.09	0.03	0.06	0.05	0.50	0.43	0.43
0 h	0.10	0.02					
3 h	0.06	0.02					
6 h	0.04	0.03					
9 h	0.07	0.01					
12 h	0.16	0.07					
Peptococcaeae, %	0.07	0.06	0.01	0.48	0.54	0.36	0.24
0 h	0.12	0.06					
3 h	0.07	0.09					
6 h	0.07	0.05					
9 h	0.06	0.08					
12 h	0.06	0.03					
Anaeroplasmataceae, %	0.12	0.22	0.02	0.03	0.54	0.01	0.59
0 h	0.16	0.23					
3 h	0.11	0.26					
6 h	0.12	0.20					
9 h	0.13	0.24					
12 h	0.10	0.18					

[1] Diets consisted of commercial concentrate (Vitality Perform 14 Horse Feed; Cargill Animal Nutrition, Elk River, MN) fed at 0.6% BW/d (LS) or 1.2% BW/d (HS; as fed basis). Remainder ad libitum access to coastal bermudagrass hay (*Cynodon dactylon*).
[2]Period = effect of period (1 and 2). Trt = effect of treatment (HS or LS). Hr = effect of time at 0, 3, 6, 9 and 12 h. Trt x Hr = interaction
[A-B] Values within a column lacking a common superscript differ by $P < 0.05$

Table A4. Percentage of sequences assigned to family over 7 d in the cecum of horses (LSMeans).

	Diet[1]		SEM	*P*-Values[2]			
Family	HS	LS		Period	Trt	D	Trt x D
UnknownFamily02, %	24.42	29.36	1.99	0.29	0.10	0.07	0.76
1 d	27.72	31.28					
2 d	22.43	29.06					
3 d	20.48	27.31					
7 d	27.04	29.80					
Paraprevotellaceae, %	20.51	17.71	0.99	0.35	0.07	0.24	0.93
1 d	21.48	18.00					
2 d	21.75	19.22					
3 d	19.37	17.67					
7 d	19.42	15.94					
Prevotellaceae, %	17.00	16.37	0.94	0.08	0.64	0.36	0.93
1 d	14.62	14.92					
2 d	17.87	16.78					
3 d	17.10	17.04					
7 d	18.42	16.73					
Lachnospiraceae, %	8.38	9.11	0.49	0.01	0.32	0.98	0.68
1 d	9.26	8.62					
2 d	8.38	9.10					
3 d	7.58	9.54					

Table A4. Continued

	Diet[1]		SEM	*P*-Values[2]			
Family	HS	LS		Period	Trt	D	Trt x D
7 d	8.31	9.19					
Porphyromonadaceae, %	3.53	5.46	0.49	0.01	0.01	0.09	0.85
1 d	4.03	5.16					
2 d	4.02	6.02					
3 d	3.44	5.88					
7 d	2.61	4.78					
Ruminococcaceae, %	3.50	3.90	0.41	0.32	0.50	0.12	0.98
1 d	2.97	3.47					
2 d	2.91	3.37					
3 d	3.61	3.83					
7 d	4.51	4.93					
Veillonellaceae, %	4.86	3.17	0.35	0.11	0.01	0.01	0.27
1 d	2.97	2.44					
2 d	4.61	3.12					
3 d	5.13	3.84					
7 d	6.75	3.29					
Spirochaetaceae, %	1.69	1.90	0.25	0.31	0.57	0.15	0.28
1 d	2.26	2.10					
2 d	2.31	1.58					
3 d	1.22	1.92					
7 d	0.96	2.00					
UnknownFamily31, %	1.43	2.05	0.21	0.36	0.06	0.01	0.01
1 d	2.16^{A}	2.25^{A}					
2 d	0.80^{B}	1.29^{B}					
3 d	1.21^{B}	2.52^{A}					
7 d	1.55^{A}	2.15^{A}					
RFP12, %	0.62	1.86	0.38	0.05	0.04	0.34	0.20
1 d	0.69	3.55					
2 d	0.75	1.51					
3 d	0.41	1.46					
7 d	0.63	0.94					
Pasteurellaceae, %	3.94	0.32	1.88	0.05	0.20	0.65	0.40

Table A4. Continued

	Diet[1]		SEM	*P*-Values[2]			
Family	HS	LS		Period	Trt	D	Trt x D
1 d	1.38	0.12					
2 d	4.18	0.19					
3 d	9.34	0.29					
7 d	0.84	0.68					
Fibrobacteraceae, %	0.91	1.08	0.15	0.15	0.46	0.01	0.13
1 d	1.49	1.20					
2 d	1.11	1.23					
3 d	0.52	1.09					
7 d	0.52	0.78					
S247, %	0.67	0.98	0.16	0.89	0.21	0.42	0.42
1 d	0.42	0.94					
2 d	0.49	1.13					
3 d	0.60	0.82					
7 d	1.19	1.04					
Clostridiaceae, %	0.66	0.70	0.04	0.01	0.60	0.33	0.22
1 d	0.64	0.62					
2 d	0.54	0.74					
3 d	0.75	0.56					
7 d	0.72	0.89					
Streptococcaceae, %	1.36	0.33	0.67	0.01	0.30	0.57	0.56
1 d	1.45	0.14					
2 d	0.94	0.27					
3 d	2.28	0.28					
7 d	0.79	0.65					
Verrucomicrobiaceae, %	0.49	0.81	0.25	0.69	0.40	0.83	0.42
1 d	0.50	0.83					
2 d	0.68	0.75					
3 d	0.48	0.81					
7 d	0.29	0.84					
UnknownFamily10, %	0.72	0.62	0.05	0.01	0.29	0.17	0.94
1 d	0.70	0.58					
2 d	0.63	0.55					
3 d	0.66	0.63					

Table A4. Continued

Family	Diet[1]		SEM	*P*-Values[2]			
	HS	LS		Period	Trt	D	Trt x D
7 d	0.88	0.74					
Succinivibrionaceae, %	1.09	0.33	0.17	0.01	0.01	0.01	0.04
1 d	0.23^{A}	0.15					
2 d	1.53$^{B.C}$	0.41					
3 d	0.54^{C}	0.47					
7 d	1.07^{B,C,D}	0.31					
UnknownFamily09, %	0.59	0.49	0.08	0.98	0.41	0.32	0.82
1 d	0.40	0.44					
2 d	0.49	0.46					
3 d	0.68	0.57					
7 d	0.78	0.48					
Bacteriodaceae, %	0.42	0.18	0.12	0.12	0.19	0.51	0.43
1 d	0.44	0.20					
2 d	0.89	0.13					
3 d	0.20	0.18					
7 d	0.15	0.20					
UnknownFamily08, %	0.44	0.40	0.04	0.08	0.63	0.15	0.25
1 d	0.46	0.33					
2 d	0.44	0.46					
3 d	0.57	0.42					
7 d	0.28	0.40					
Flavobacteriaceae, %	0.63	0.20	0.23	0.09	0.22	0.73	0.42
1 d	1.24	0.13					
2 d	0.36	0.20					
3 d	0.56	0.15					
7 d	0.35	0.34					
Desulfovibrionaceae, %	0.19	0.28	0.04	0.57	0.18	0.04	0.31
1 d	0.21	0.43					
2 d	0.16	0.19					
3 d	0.23	0.24					
7 d	0.17	0.25					
Coprobacillaceae, %	0.29	0.22	0.06	0.02	0.41	0.46	0.34
1 d	0.55	0.16					

Table A4. Continued

	Diet[1]		SEM	*P*-Values[2]			
Family	HS	LS		Period	Trt	D	Trt x D
2 d	0.20	0.20					
3 d	0.20	0.34					
7 d	0.21	0.17					
Erysipelotrichaceae, %	0.24	0.30	0.03	0.01	0.31	0.04	0.16
1 d	0.22	0.23					
2 d	0.21	0.37					
3 d	0.24	0.26					
7 d	0.30	0.33					
Lactobacillaceae, %	0.22	0.09	0.06	0.13	0.15	0.50	0.97
1 d	0.26	0.09					
2 d	0.18	0.06					
3 d	0.22	0.10					
7 d	0.21	0.09					
Anaeroplasmataceae, %	0.12	0.20	0.02	0.12	0.07	0.98	0.90
1 d	0.12	0.20					
2 d	0.13	0.22					
3 d	0.14	0.18					
7 d	0.11	0.20					
UnknownFamily04, %	0.11	0.17	0.03	0.03	0.24	0.14	0.02
1 d	$0.11^{A,C}$	$0.23^{A,C,D}$					
2 d	0.13^{B}	$0.11^{B,D}$					
3 d	0.10^{C}	0.21^{C}					
7 d	$0.11^{A,B,C}$	0.13^{D}					

[1] Diets consisted of commercial concentrate (Vitality Perform 14 Horse Feed; Cargill Animal Nutrition, Elk River, MN) fed at 0.6% BW/d (LS) or 1.2% BW/d (HS; as fed basis). Remainder ad libitum access to coastal bermudagrass hay (*Cynodon dactylon*).
[2]Period = effect of period (1 and 2). Trt = effect of treatment (HS or LS). Day = effect of time at 1, 2, 3, and 7 d. Trt x Day = interaction
[A-D] Values within a column lacking a common superscript differ by $P < 0.05$

Table A5. Percentage of sequences assigned to genus over first 12 h in the cecum of horses (LSMeans).

	Diet[1]		SEM	*P*-Values[2]			
Genus	HS	LS		Period	Trt	Hr	Trt x Hr
Prevotella, %	14.81	15.42	1.29	0.78	0.74	0.03	0.88
0 h	13.92	14.50					
3 h	13.56	13.15					
6 h	14.48	15.07					
9 h	15.54	16.10					
12 h	16.54	18.28					
YRC22, %	7.35	5.26	0.95	0.18	0.14	0.33	0.95
0 h	7.41	5.75					
3 h	9.05	6.31					
6 h	7.21	5.31					
9 h	6.35	4.25					
12 h	6.72	4.66					
CF231, %	6.33	6.56	0.77	0.74	0.83	0.02	0.34
0 h	6.39	7.77					
3 h	7.48	7.07					
6 h	5.93	6.54					
9 h	6.31	6.48					
12 h	5.53	4.99					
Prevotella1, %	4.02	3.03	0.84	0.82	0.41	0.69	0.86
0 h	3.99	2.33					
3 h	3.79	2.50					
6 h	3.55	2.69					
9 h	4.17	3.06					
12 h	4.82	4.57					
Treponema, %	2.61	1.88	0.27	0.99	0.08	0.80	0.54
0 h	2.18	1.91					
3 h	2.40	1.67					
6 h	2.24	2.11					
9 h	3.29	2.03					
12 h	2.93	1.67					
Paludibacter, %	1.75	2.33	0.24	0.01	0.11	0.01	0.63
0 h	2.63	2.19					
3 h	0.87	1.47					
6 h	1.16	2.10					
9 h	2.15	3.23					

Table A5. Continued

Genus	Diet[1]		SEM	*P*-Values[2]			
	HS	LS		Period	Trt	Hr	Trt x Hr
12 h	1.91	2.66					
Phascolarctobacterium, %	1.70	1.64	0.16	0.64	0.79	0.24	0.30
0 h	1.25	1.48					
3 h	1.61	1.75					
6 h	1.75	1.58					
9 h	1.88	1.87					
12 h	2.01	1.51					
Anaerovibrio,%	0.88	1.02	0.19	0.17	0.62	0.04	0.82
0 h	0.32	0.55					
3 h	0.33	0.61					
6 h	1.18	0.80					
9 h	1.03	1.06					
12 h	1.56	2.10					
Roseburia, %	1.34	1.40	0.30	0.90	0.88	0.56	0.33
0 h	1.06	1.44					
3 h	1.11	1.19					
6 h	2.04	1.09					
9 h	1.13	1.01					
12 h	1.35	2.28					
Fibrobacter, %	1.46	1.28	0.23	0.10	0.60	0.05	0.82
0 h	1.63	1.56					
3 h	1.14	1.23					
6 h	1.51	1.18					
9 h	1.84	1.42					
12 h	1.16	1.00					
Streptococcus, %	0.86	0.32	0.24	0.35	0.14	0.01	0.37
0 h	0.13	0.08					
3 h	0.12	0.17					
6 h	0.14	0.10					
9 h	0.79	0.59					
12 h	1.79	0.65					
Akkermansia, %	0.64	0.77	0.30	0.51	0.78	0.05	0.67
0 h	0.66	0.68					

Table A5. Continued

	Diet[1]		SEM	*P*-Values[2]			
Genus	HS	LS		Period	Trt	Hr	Trt x Hr
3 h	1.13	0.76					
6 h	0.50	0.84					
9 h	0.26	0.66					
12 h	0.68	0.90					
Coprococcus, %	0.69	0.71	0.06	0.06	0.81	0.01	0.63
0 h	0.98	0.82					
3 h	0.93	0.92					
6 h	0.55	0.72					
9 h	0.54	0.56					
12 h	0.45	0.53					
Ruminococcus, %	0.39	0.39	0.03	0.03	0.98	0.01	0.85
0 h	0.47	0.46					
3 h	0.48	0.44					
6 h	0.37	0.40					
9 h	0.33	0.31					
12 h	0.31	0.34					
Bacteroides, %	0.61	0.28	0.37	0.19	0.54	0.62	0.19
0 h	0.92	0.13					
3 h	0.51	0.10					
6 h	0.37	0.14					
9 h	0.59	0.12					
12 h	0.66	0.90					
Clostridium, %	0.34	0.41	0.05	0.01	0.37	0.01	0.39
0 h	0.36	0.58					
3 h	0.57	0.54					
6 h	0.30	0.31					
9 h	0.23	0.35					
12 h	0.23	0.28					
Oscillospira, %	0.31	0.26	0.03	0.19	0.26	0.01	0.66
0 h	0.22	0.16					
3 h	0.26	0.19					
6 h	0.26	0.26					
9 h	0.43	0.42					
12 h	0.39	0.25					

Table A5. Continued

	Diet[1]		SEM	*P*-Values[2]			
Genus	HS	LS		Period	Trt	Hr	Trt x Hr
Desulfovibrio, %	0.23	0.41	0.06	0.84	0.08	0.22	0.31
0 h	0.22	0.56					
3 h	0.35	0.48					
6 h	0.21	0.44					
9 h	0.19	0.29					
12 h	0.19	0.31					

[1] Diets consisted of commercial concentrate (Vitality Perform 14 Horse Feed; Cargill Animal Nutrition, Elk River, MN) fed at 0.6% BW/d (LS) or 1.2% BW/d (HS; as fed basis). Remainder ad libitum access to coastal bermudagrass hay (*Cynodon dactylon*).
[2]Period = effect of period (1 and 2). Trt = effect of treatment (HS or LS). Hr = effect of time at 0, 3, 6, 9 and 12 h. Trt x Hr = interaction

Table A6. Percentage of sequences assigned to genus over 7 d in the cecum of horses (LSMeans).

	Diet[1]		SEM	*P*-Values[2]			
Genus	HS	LS		Period	Trt	D	Trt x D
Prevotella, %	17.00	16.37	0.95	0.08	0.64	0.36	0.93
1 d	14.62	14.92					
2 d	17.87	16.78					
3 d	17.10	17.03					
7 d	18.42	16.73					
YRC22, %	6.82	4.55	0.70	0.38	0.04	0.74	0.56
1 d	7.16	5.36					
2 d	6.45	5.13					
3 d	6.41	3.86					
7 d	7.24	3.85					
CF231, %	4.95	5.96	0.45	0.38	0.14	0.43	0.67
1 d	5.95	6.52					
2 d	4.76	6.15					
3 d	4.83	5.31					
7 d	4.27	5.88					
Prevotella1, %	3.67	3.74	0.44	0.74	0.17	0.26	0.36
1 d	3.35	2.69					
2 d	5.28	2.93					
3 d	3.23	3.12					
7 d	2.83	2.23					
Treponema, %	1.69	1.90	0.25	0.31	0.57	0.15	0.28
1 d	2.26	2.10					
2 d	2.31	1.58					
3 d	1.22	1.92					
7 d	0.96	2.00					
Paludibacter, %	1.15	2.28	0.17	0.01	0.01	0.34	0.75
1 d	1.15	2.12					
2 d	1.50	2.31					
3 d	1.10	2.49					
7 d	0.84	2.22					
Phascolarctobacterium, %	2.22	1.73	0.18	0.58	0.08	0.01	0.30
1 d	1.76	1.57					
2 d	1.92	1.70					

Table A6. Continued

	Diet[1]		SEM	*P*-Values[2]			
Genus	HS	LS		Period	Trt	D	Trt x D
3 d	2.26	1.72					
7 d	2.93	1.91					
Anaerovibrio, %	2.54	1.40	0.32	0.01	0.03	0.01	0.47
1 d	1.11	0.86					
2 d	2.63	1.36					
3 d	2.75	2.09					
7 d	3.65	1.31					
Roseburia, %	1.59	0.96	0.29	0.01	0.15	0.86	0.62
1 d	1.98	1.15					
2 d	1.89	0.91					
3 d	1.26	0.94					
7 d	1.25	0.86					
Fibrobacter, %	0.91	1.08	0.15	0.15	0.46	0.01	0.13
1 d	1.49	1.20					
2 d	1.11	1.23					
3 d	0.52	1.09					
7 d	0.52	0.78					
Streptococcus, %	1.35	0.32	0.66	0.01	0.30	0.56	0.56
1 d	1.42	0.13					
2 d	0.92	0.26					
3 d	2.27	0.28					
7 d	0.79	0.63					
Akkermansia, %	0.49	0.81	0.25	0.69	0.40	0.83	0.42
1 d	0.50	0.83					
2 d	0.68	0.75					
3 d	0.48	0.81					
7 d	0.29	0.84					
Coprococcus, %	0.58	0.70	0.05	0.49	0.14	0.27	0.50
1 d	0.55	0.73					
2 d	0.64	0.62					
3 d	0.46	0.63					
7 d	0.67	0.84					
Ruminococcus, %	0.35	0.40	0.03	0.01	0.32	0.79	0.82

Table A6. Continued

	Diet[1]		SEM	*P*-Values[2]			
Genus	HS	LS		Period	Trt	D	Trt x D
1 d	0.36[A]	0.40					
2 d	0.32[B]	0.42					
3 d	0.34[A]	0.33					
7 d	0.36[A]	0.44					
Clostridium, %	0.28	0.35	0.04	0.05	0.27	0.57	0.01
1 d	0.31	0.30					
2 d	0.18	0.39					
3 d	0.33	0.27					
7 d	0.31	0.45					
Oscillospira, %	0.32	0.33	0.04	0.25	0.87	0.13	0.98
1 d	0.25	0.27					
2 d	0.30	0.32					
3 d	0.41	0.40					
7 d	0.31	0.32					
Desulfovibrio, %	0.19	0.28	0.04	0.57	0.18	0.04	0.31
1 d	0.21	0.43					
2 d	0.16	0.19					
3 d	0.23	0.24					
7 d	0.17	0.25					

[1] Diets consisted of commercial concentrate (Vitality Perform 14 Horse Feed; Cargill Animal Nutrition, Elk River, MN) fed at 0.6% BW/d (LS) or 1.2% BW/d (HS; as fed basis). Remainder ad libitum access to coastal bermudagrass hay (*Cynodon dactylon*).
[2]Period = effect of period (1 and 2). Trt = effect of treatment (HS or LS). Day = effect of time at 1, 2, 3, and 7 d. Trt x Day = interaction
[A-B] Values within a column lacking a common superscript differ by $P < 0.05$